Katholieke Universiteit Leuven
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IDENTIFICATION OF POTENTIAL TARGETS FOR VACCINATION AGAINST *STAPHYLOCOCCUS EPIDERMIDIS* BIOFILMS

Mohammad Shahrooei

Doctoral thesis in Medical Sciences

Leuven, 2010

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LIST OF ABBREVIATIONS

aa	amino acid
Aap	accumulation-associated protein
agr	accessory-gene regulator
AI	autoinducer
ANOVA	analysis of variance
AtlE	autolysin E (E indicates its origin in *S. epidermidis*)
Bap	biofilm-associated protein
BHI	brain heart infusion
Bhp	bap homologue protein
BSA	bovine serum albumin
cat194	chloramphenicol acetyltransferase-encoding gene of pC194 for chloramphenicol resistance
CFA	complete Freund's adjuvant
CFU	colony forming unit
ClfA	clumping factor A
Cn	collagen
CNS	coagulase-negative *Staphylococcus* spp.
CRBSI	catheter-related bloodstream infection
CRI	catheter-related infection
CVC	central venous catheter
DNA	deoxyribonucleic acid
Ebps	elastin-binding protein of *Staphylococcus aureus*
ECM	extracellular matrix
EGF	ex-germfree
Embp	extracellular matrix binding protein

ermC	ribosomal methylase-encoding gene of pE194 for erythromycin resistance
FAM	6-carboxy-fluorescein
Fbe	fibrinogen-binding protein of *S. epidermidis*
FBI	foreign body infection
Fg	fibrinogen
FITC	fluorescein-isothiocyanate
Fn	fibronectin
GehD	a *S. epidermidis* lipase protein
gmk	guanylate monokinase
HSL	homoserine lactones
ica	intercellular adhesion
IFA	incomplete Freund's adjuvant
IPTG	isopropyl-beta-d-thiogalactopyranoside
IS	insertion sequence
mec	methicillin resistance determinant
MMLV	moloney murine leukemia virus
MSCRAMM	microbial surface components recognizing adhesive matrix molecules
NCBI	National Centre of Biotechnology Information
NICU	neonatal intensive care unit
NVE	native valve endocarditis
P*blaZ*	constitutive β-lactamase promoter module
PBS	phosphate buffered saline
PIA	polysaccharide intercellular adhesin
PNAG	poly-*N*-acetylglucosamine
PSM	phenol-soluble modulin
PVE	prosthetic valve endocarditis
QS	quorum-sensing
RIP	RNAIII-inhibiting peptide
RNA	ribonucleic acid

rSes	recombinant Ses
sae	*S. aureus* exoprotein expression
sarA	staphylococcal accessory regulator A
SdrF, G, H	serine-aspartate repeat protein family (F, G, H)
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Ses	*Staphylococcus epidermidis* surface-exposed protein
SigB	sigma factor sigma B
sir	staphylococcal iron regulator
sit	staphylococcal iron transport
spp	species (plural)
Ssp-1, 2	staphylococcal surface proteins 1, 2
TAMRA	6-carboxy-tetramethyl-rhodamine
TetR	tetracycline transcriptional regulators
TSA	tryptone soya agar
UDP	uridine diphosphate
UTI	urinary tract infection
UV	ultraviolet
Vn	vitronectin
vol/vol	volume volume ratio
VWF	von Willebrand factor
WC	whole cell
wt/vol	weight volume ratio
σ^B	alternative sigma factor sigma B (SigB)

SUMMARY

Compared with S*taphylococcus aureus*, *S. epidermidis* does not produce as many toxins and tissue-damaging exoenzymes and *S. epidermidis* infections may rarely become life-threatening. However, *S. epidermidis* has gained substantial interest in recent years because it is now considered as the major cause of device-related infections, infections which have increased in number, owing to the increased use of such devices.

The ability to form biofilms on medical implant surfaces is considered the main virulence factor of *S. epidermidis*. Biofilms are notoriously resistant to both immune system attack and antimicrobial agents. Currently, the only completely effective method for curing biofilm infections is to remove the infected device, a risky, costly and stressful procedure.

Different strategies are used to combat biofilm infections. The traditional approach to prevent biofilm formation is local administration of bactericidal agents. However, compared to their planktonic counterparts, bacteria within a biofilm are up to a 1000 times more resistant to antimicrobial agents. Immunoprophylaxis and immunotherapy targeting proteins and surface components that are expressed *in vivo* and are important for biofilm formation are promising new approaches to prevent and treat biofilms. Antibodies against extracellular macromolecules and surface binding proteins essential for cell-surface and cell-cell interaction and adhesion, such as PIA, teichoic acids, Fbe and Aap and vaccination of rats with purified PIA/PNAG have been shown to prevent biofilm formation. An advantage of immunotherapeutic methods is the lesser risk for the development of resistance.

In this study, using a new approach called “reverse vaccinology” we tried to identify new potential targets for immunotherapy against *S. epidermidis* biofilms. The first step in reverse vaccinology is the identification of the complete repertoire of antigens that are expressed on the surface. Using an *in silico* procedure, 57 proteins have been

identified as *S. epidermidis* 'surface-exposed proteins' (Ses) of which 32 were annotated as (conserved) hypothetical. From these, 13 proteins were selected based on protein size, number of antigenic determinants and the role in *S. epidermidis* biofilm formation and pathogenesis of the protein family to which the candidate protein belongs.

The presence of genes coding for the 13 selected proteins (*ses* genes) in clinical and commensal isolates of *S. epidermidis* was investigated by PCR. Using the previously described *in vitro* and *in vivo* rat models for foreign body infections (FBI) (Vandecasteele *et al.* 2001;Vandecasteele *et al.* 2002), the expression of selected genes was investigated in planktonic and sessile bacteria during FBI. Seven genes were present in all tested isolates, whereas 6 genes were absent in some of the isolates. The *in vitro* and *in vivo* expression patterns of *ses* genes varied widely.

Out of 13 selected proteins, 5 proteins of which the function had not yet been characterized include 3 LPXTG proteins and the 2 largest ABC transporters were selected for the *in vitro* biofilm inhibition studies. The extracellular parts of these 5 selected Ses proteins were cloned, expressed, purified and polyclonal rabbit antibodies against them were raised. Using ELISA and western blotting techniques, expression of the selected 5 proteins on the surface of *S. epidermidis* cells was investigated. Total IgGs were isolated from pre- and hyperimmune sera and used in a semi-quantitative microtiter plate method to evaluate the effect of different anti-Ses IgGs on *S. epidermidis* biofilm formation. It was shown that these proteins are surface-exposed, except for SesK and SesM which might be surface-exposed proteins which expressed under specific, unknown circumstances. Maximum inhibition of initial attachment and overnight biofilm formation on polystyrene surface was achieved by the total IgGs purified from serum of the rabbit immunized with rSesC.

SesC, against which antibodies exhibited the highest inhibition of biofilm formation *in vitro,* was selected for further investigation. Specific anti-SesC IgGs (αSesC-IgGs) were isolated from total IgGs isolated from hyper immune serum of rabbit immunized with rSesC using antigen-affinity purification. Conventional fluorescence microscopy was used to confirm SesC protein expression on the surface of sessile and planktonic *S. epidermidis* bacteria *in vitro*. The effect of different concentrations of αSesC-IgGs was tested *in vitro* on primary attachment, overnight biofilm formation and

established (1-day old) biofilms of different *S. epidermidis* strains. We also tested the effect of αSesC-IgGs on established biofilms of *S. epidermidis* in the *in vivo* rat model for FBI. Finally, rats were immunized with rSesC and the effect of immunization on FBI was evaluated. *In vitro* experiments showed that rabbit polyclonal αSesC-IgGs could significantly reduce initial attachment, and prevent development of early and established biofilms of *S. epidermidis* on the abiotic surface of polystyrene plates. *In vivo* experiments showed the efficiency of active and passive immunization against SesC on *S. epidermidis* biofilm formation on the polyurethane catheters.

To unravel the structure and function of SesC, different bioinformatics tools were used and we studied the effect of expression and overexpression of *sesC* in the *sesC*-negative strain *S. aureus* RN4220 and in the *sesC*-positive strain *S. epidermidis* RP62A on biofilm formation and adherence to host matrix proteins. We showed that *S. aureus* RN-transformants expressing SesC can bind fibrinogen (Fg) but not other extracellular matrix (ECM) proteins better than their parental strain and that αSesC-IgGs could significantly reduce the Fg-binding ability of RN-transformants expressing SesC and of the RP62A wild type and transformants.

In conclusion, our findings revealed that SesC represents a promising target for prevention and treatment of *S. epidermidis* biofilms by affecting primary attachment, biofilm accumulation and detachment. SesC might be a potential Fg-binding MSCRAMM (microbial surface components recognizing adhesive matrix molecules), which plays a role in attachment to both biotic and abiotic surfaces. However, further investigation is necessary to characterize the structure and identify the precise role of SesC in biofilm formation and the mechanism of function of αSesC-IgG antibodies *in vivo*.

SAMENVATTING

Vergeleken met *Staphylococcus aureus*, is *S. epidermidis* een species die veel minder weefselbeschadigende toxines en exo-enzymen produceert. *S. epidermidis* infecties zijn ook zelden levensbedreigend. *S. epidermidis* geniet recent echter stijgende belangstelling, omdat deze stafylokok nu wordt beschouwd als de belangrijkste oorzaak van vreemdlichaaminfecties, die wegens het toenemend gebruik van biomaterialen zijn toegenomen.

De mogelijkheid om een biofilm te vormen op medische implantaatoppervlakken wordt beschouwd als de belangrijkste virulentiefactor van *S. epidermidis*. Biofilmen zijn beduidend minder gevoelig voor antibiotica. De enige echt efficiënte methode om biofilminfecties te genezen, is het besmette vreemdlichaam te verwijderen, een risicovolle, moeilijke en dure procedure.

Verschillende strategieën worden gebruikt ter bestrijding van biofilminfecties. Lokaal of systemisch gebruik van bactericide agentia is de traditionele aanpak om biofilmvorming te voorkomen of te genezen. In vergelijking met hun planktonische tegenhangers, zijn bacteriën in een biofilm echter tot duizend keer beter bestand tegen antimicrobiële agentia. Immuunprofylaxis gericht tegen eiwitten die deel uitmaken van het celoppervlak en die tot expressie komen '*in vivo*' en bijgevolg belangrijk zijn voor de vorming van biofilm is een veelbelovende nieuwe benadering voor de preventie en behandeling van biofilmen. Er werd reeds aangetoond dat antilichamen tegen extracellulaire macromoleculen zoals PIA, teichoïnezuren, Fbe en Aap, die essentieel zijn voor de interactie tussen het bacteriële celoppervlak en het vreemdlichaam en tussen bacteriën in de biofilm onderling, en vaccinatie van ratten met gezuiverde PIA/PNAG, biofilmvorming kunnen voorkomen. Een voordeel van immuuntherapeutische methoden is het verminderde risico op de ontwikkeling van resistentie.

In deze studie hebben we via "reverse vaccinology" geprobeerd nieuwe potentiële doelen voor immuuntherapie tegen *S. epidermidis* biofilmen te identificeren. De eerste stap in deze 'reverse vaccinology' is de identificatie van het volledige repertoire van antigenen die op het oppervlak tot expressie worden gebracht. Met behulp van een '*in silico*' procedure, werden 57 eiwitten geïdentificeerd als oppervlakteeiwitten van *S. epidermidis* (Ses) waarvan 32 eiwitten geannoteerd zijn als eiwitten met ongekende functie. Uit deze groep werden 13 eiwitten geselecteerd op basis van grootte, aantal antigene determinanten en de rol in *S. epidermidis* biofilmvorming en pathogenese van de eiwitgroep waartoe het kandidaat-eiwit behoort.

Na de '*in silico*' selectie van potentiële vaccinantigenen, is de volgende stap het valideren van de geselecteerde proteïnen via analyse van de expressie '*in vitro*' en '*in vivo*'. De aanwezigheid van deze 13 genen in klinische en commensale isolaten van *S. epidermidis* werd onderzocht en - met onze eerder beschreven '*in vitro*' en '*in vivo*' ratmodellen voor vreemdlichaaminfecties – werd de expressie van deze 13 genen onderzocht in planktonische en sessiele bacteriën tijdens de vreemdlichaaminfecties.

De expressiepatronen '*in vitro*' en '*in vivo*' van de *ses* genen zijn zeer gevarieerd. Daarom, op basis van de hierboven genoemde kenmerken, werden 5 van deze 13 eiwitten geselecteerd; 3 eiwitten met een LPXTG sequentie en een niet-gekende rol in biofilmvorming en de twee grootste ABC transportereiwitten, eveneens met ongekende functie. Het extracellulaire deel van deze 5 geselecteerde Ses eiwitten werd gekloond, tot expressie gebracht en gezuiverd en polyklonale antilichamen tegen deze eiwitten werden opgewekt in konijnen. De antilichamen werden geïsoleerd uit pre- en hyperimmuun sera en deze werden gebruikt in een semi-kwantitatieve microtiterplaat assay voor het evalueren van het effect van verschillende anti-Ses-antilichamen op *S. epidermidis* biofilmvorming.

De hoogste remming van de '*in vitro*' biofilmvorming werd gevonden voor de anti-SesC antilichamen. SesC werd daarom voor nader onderzoek geselecteerd. Specifieke anti-SesC antilichamen werden geïsoleerd uit de totale antilichaamfractie uit hyperimmuun serum van konijnen via affiniteitschromatografie met recombinant SesC. Conventionele fluorescentiemicroscopie werd gebruikt om de expressie van SesC op het oppervlak van sessiele en planktonische *S. epidermidis* bacteriën '*in vitro*' te bevestigen.

Het effect van verschillende concentraties van anti-SesC antilichamen '*in vitro*' op primaire aanhechting, biofilmvorming tijdens overnacht incubatie en op mature (1-dag oude) biofilmen van verschillende *S. epidermidis* stammen werd getest. We testten ook het effect van anti-SesC antilichamen op mature biofilmen van *S. epidermidis* in het '*in vivo*' rat model voor vreemdlichaaminfecties. Tot slot hebben we ook ratten geïmmuniseerd met SesC en het effect van deze immunisatie op vreemdlichaaminfecties bij ratten geëvalueerd.

Om de structuur en de functie van SesC te ontrafelen, werden verschillende bio-informatica hulpmiddelen gebruikt en hebben we het effect bestudeerd op biofilmvorming en aanhechting aan gastheermatrixeiwitten van de expressie van SesC in de *sesC*-negatieve *S. aureus* RN4220 en het effect van overexpressie in de *sesC*-positieve *S. epidermidis* RP62A. Er werd aangetoond dat de *S. aureus* RN-transformanten die SesC tot expressie brengen beter binden aan fibrinogeen maar niet aan andere extracellulaire matrixproteïnen. Bovendien konden we observeren dat anti-SesC antilichamen deze binding significant verminderden.

Uit onze bevindingen blijkt dat SesC een veelbelovend doelwit is voor preventie en behandeling van *S. epidermidis* biofilmen via interactie met de primaire aanhechting, accumulatie en loslating. SesC is mogelijk een fibrinogeenbindende MSCRAMM (microbial surface components recognizing adhesive matrix molecules) en speelt een rol in aanhechting aan zowel biotische als abiotische oppervlakken. Verder onderzoek is echter noodzakelijk om de structuur en de precieze rol van SesC in biofilmvorming te karakteriseren alsook het werkingsmechanisme van de anti-SesC antilichamen *in vivo*.

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CHAPTER 1:

GENERAL INTRODUCTION

1. General introduction

1.1. The genus *Staphylococcus*

In 1878, Robert Koch was the first to describe *Staphylococcus* spp. in human pus. In 1880, Pasteur cultivated *Staphylococcus* spp. in a liquid medium. Alexander Ogston named them and demonstrated that they were pathogenic, causing acute and chronic abscesses in mice and guinea pig. In 1884, Rosenbach described the two pigmented colony types of *Staphylococcus* spp. and proposed the appropriate nomenclature: *S. aureus* (yellow) and *S. albus* (white) which was subsequently changed to *S. epidermidis*.

Staphylococcus spp. are non-motile, non-spore forming, Gram-positive spherical bacteria about 1 micrometer in diameter that are members of the bacterial family *Staphylococcaceae*. *Staphylococcus* spp. are capable of growth both aerobically and anaerobically (facultative anaerobes). All species can grow in the presence of bile salts, and are catalase positive.

Staphylococcus spp. are normal inhabitants of the skin and mucous membranes of humans and other organisms (Piette and Verschraegen 2009). So far, more than 40 species and subspecies in the genus *Staphylococcus* spp. have been identified (Trulzsch *et al.* 2007). This genus is divided into two groups according to production of enzyme coagulase that coagulates plasma: coagulase-positive, including *S. aureus* and *S. intermedius*; and numerous coagulase-negative *Staphylococcus* (CNS) species.

Currently, there are more than 40 recognized species of CNS. Of 40 CNS species validly published, about 16 species are indigenous in humans; including *S. cohnii*, *S. saprophyticus*, *S. sciuri*, *S. xylosis*, *S. auricularis*, *S. capitis*, *S. caprae*, *S. epidermidis*, *S. haemolyticus*, *S. hominis*, *S. lugdunensis*, *S. pasteuri*, *S. saccharolyticus*, *S. schleiferi*, *S. simulans*, *S. warneri* (von Eiff *et al.* 2001).

1.2. Coagulase-negative staphylococci infections

Compared with *S. aureus*, the most common cause of staphylococcal infections, CNS do not produce as many toxins and tissue-damaging exoenzymes and have long

been regarded as apathogenic and harmless commensals of man (Akiyama *et al.* 2000;Huebner and Goldmann 1999;Vuong and Otto 2002). CNS are now considered as an important causative agents of nosocomial bacteremias, infections of intravascular catheters and indwelling devices especially in neonates, immunocompromised and seriously ill patients, osteomyelitis, endocarditis, and wound infection following cardiothoracic surgery in the absence of prostheses (Huebner and Goldmann 1999;Knausz *et al.* 2005;Otto 2004b;Piette and Verschraegen 2009;Rogers *et al.* 2009;Schulin and Voss 2001;von Eiff *et al.* 2002). Among the CNS, *S. epidermidis* is the most frequently isolated, making up three-fourths of the CNS recovered from clinical specimens and accounts for approximately 75% of the infections caused by CNS.

1.3. Clinical importance of *S. epidermidis*

For the following reasons, *S. epidermidis* is now considered as a commensal opportunistic pathogen and has gained substantial interest in recent years;

- *S. epidermidis* is the major pathogen of the CNS category and comprises 65-90% of all *Staphylococcus* spp. recovered from human sources (Huebner and Goldmann 1999).
- Approximately 180 million peripheral intravascular catheters and 7 million central venous catheters (CVC) are used annually in the United States alone (Raad *et al.* 2000). Intravascular catheter-related infection (CRI) ranges widely in the literature from 1% to 2% to greater than 25% (Eggimann *et al.* 2004). It is estimated that approximately 250,000 cases of intravascular catheter-related bloodstream infections (CRBSI) occur yearly in the United States alone (Rogers *et al.* 2009). *S. epidermidis* is responsible for 50-70% of these infections (Huebner and Goldmann 1999).
- Prosthetic valve endocarditis (PVE) and native valve endocarditis (NVE) are caused by CNS in 15-50% and 5-8% of cases, respectively. *S. epidermidis* is the most prominent cause of both PVE and NVE (Chu *et al.* 2008;Huebner and Goldmann 1999;Lalani *et al.* 2006;Rogers *et al.* 2009).

- CNS are the predominant pathogens causing 48% to 67% of cerebrospinal fluid shunt infections and *S. epidermidis* is the most common bacterial species isolated from these infections (Roos 1997).
- Together with *S. aureus, S. epidermidis* ranks second as cause of surgical site infection (Rogers *et al.* 2009;Wilson *et al.* 1988).
- CNS are responsible for two types of urinary tract infection (UTI): infections caused by *S. saprophyticus*, which affect young (18 to 35 years), sexually active women and young female outpatients; and UTIs due to mainly *S. epidermidis*, which occur equally in men and women (Huebner and Goldmann 1999;Rogers *et al.* 2009).
- CNS are currently responsible for more than 50% of all nosocomial infections in neonatal intensive care units (NICU). *S. epidermidis* is the most common isolate and has been implicated in 34% of cases of CRIs in children and 51% in neonates (Hira *et al.* 2007;Rogers *et al.* 2009).

Despite all these problems, Michael Otto in a recently published review paper in Nature Review Microbiology (Otto 2009) listed "evidences which indicated that most 'virulence factors' of *S. epidermidis* originally had roles in the commensal lifestyle of this species" and drew the conclusion that "*S. epidermidis* should be regarded as an 'accidental' pathogen, the clinical importance of which stems less from a dedicated infectious lifestyle and more from the frequency of contamination events and the existence of mechanisms, such as adhesion and immune evasion, that are beneficial for the bacteria during both colonization and chronic infection".

1.4. *S. epidermidis* device-related infections

More than half a billion medical devices, ranging from simple catheters to total artificial hearts, are implanted in patients every year. An estimated 11 million people in the United States alone have medical implants, indicating the importance and value of implants. These devices, although helping millions of people to stay active, are prone to infection and prompt the bacteria to colonize. Colonization of foreign bodies by bacteria lead to serious local and systemic infections called foreign body infections (FBI).

Bacteria can contaminate a medical device during surgical implant insertion or through hematogenous seeding (Figure 1.1.). Once attached, bacteria start producing biofilm.

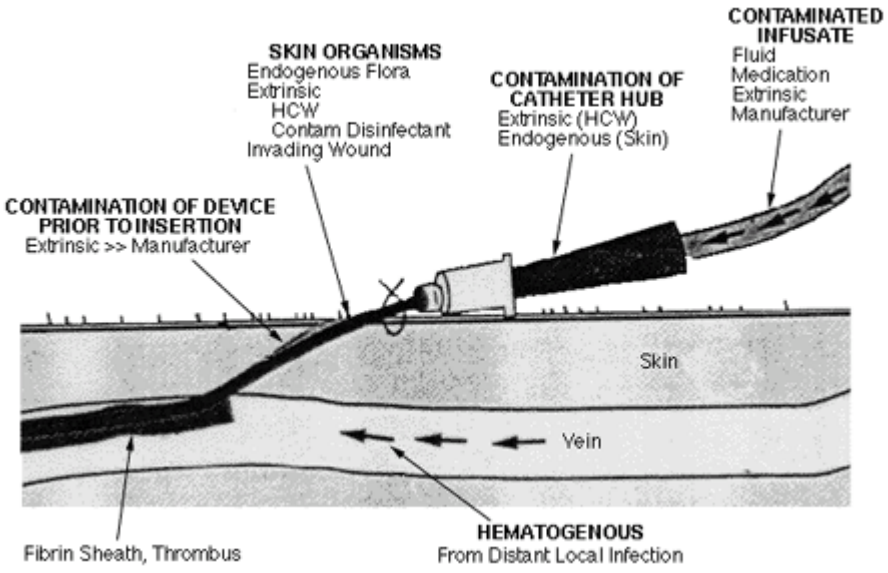


Figure 1.1. Potential sources of catheter-related bloodstream infections. (http://www.phac-aspc.gc.ca/publicat/ccdr-rmtc/97vol23/23s8/images/fig1inte.gif). **HCW:** Healthcare worker, **Contam:** contaminated

A biofilm is defined as "a structured community of bacterial cells enclosed in a self-produced polymeric matrix and adherent to an inert or living surface" (Costerton *et al.* 1999). Biofilms are notoriously resistant to immune system attack and antimicrobial agents. They are a huge problem in medicine and industry, responsible for ≈65% of all bacterial infections (del Pozo and Patel 2007).

S. epidermidis as a permanent and ubiquitous colonizer of human skin can easily contaminate devices during insertion. In fact, the success of *S. epidermidis* as a pathogen is mainly attributed to its ability to adhere to surfaces and form biofilm. The costs related to vascular CRBSIs alone caused by *S. epidermidis* amount to more than US$2 billion annually in the United States (Otto 2009;Rogers *et al.* 2009). *S. epidermidis* represents the most common source of infections on indwelling medical devices (Otto 2009).

1.5. *S. epidermidis* biofilm formation; switch from commensalism to pathogenicity

Except for the rare infections such as osteomyelitis, endocarditis and wound infections in the absence of prostheses, CNS and, in particular *S. epidermidis* infections mostly occur in association with the use of medical devices. The ability to form biofilms on plastic devices is a major virulence factor for *S. epidermidis* (Raad *et al.* 1998).

The development of a biofilm occurs in several steps. Biofilm formation starts with the initial adhesion of cells to a surface and their subsequent maturation phase. Detachment of cells or cell clusters from an existing biofilm can lead to the dissemination of the infection (Figure 1.2.).

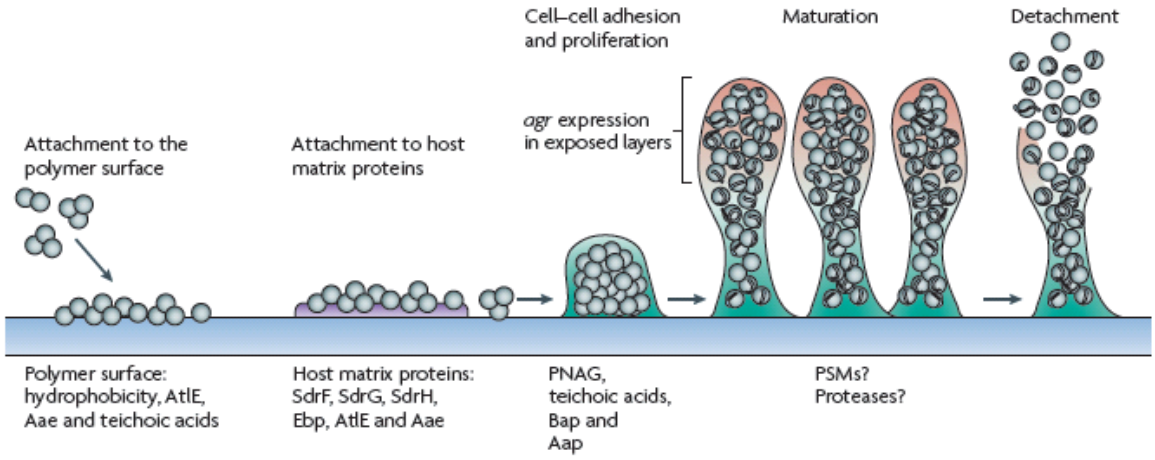


Figure 1.2. Schematic overview of the different stages of *S. epidermidis* biofilm formation (Otto 2009).
PSMs: phenol-soluble modulins

1.5.1. Primary attachment

Initial attachment can be based on direct binding to the abiotic surface of an implant. This process is mediated by hydrophobic interaction, van der Waals forces, electrostatic interaction between bacteria and implant surface, and several surface proteins, e.g. Ssp-1, Ssp-2, Bhp, and AtlE and teichoic acids (Gross *et al.* 2001;Heilmann *et al.* 1997;Tormo *et al.* 2005a;Veenstra *et al.* 1996). Alternatively, indirect binding to the

surface of implanted medical devices coated with host plasma and matrix proteins (conditioned material) such as fibrinogen (Fg), fibronectin (Fn), collagen (Cn), vitronectin (Vn) and elastin can be mediated via microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) such as SdrG (Fbe), Embp, GehD, AtlE, and Ebps, respectively (Bowden *et al.* 2002;Davis *et al.* 2001;Heilmann *et al.* 1997;Nilsson *et al.* 1998;Park *et al.* 1996;Williams *et al.* 2002).

1.5.2. Accumulation

Following adherence to the surfaces of implants, biofilms develop through intercellular aggregation that is mediated by intercellular adhesins. These include surface macromolecules such as exopolysaccharide, surface proteins, teichoic acids and extracellular DNA originating from lysed cells which are involved in formation of extracellular biofilm matrix.

In *Staphylococcus* spp., production of polysaccharide intercellular adhesin [PIA, also called poly-*N*-acetylglucosamine (PNAG)] by the genes in the intercellular adhesion (*ica*) operon, is currently the best-understood mechanism [*ica* or PIA-dependent mechanism (Figure 1.3.a.)] of biofilm formation (Cramton *et al.* 1999;Gerke *et al.* 1998;Mack *et al.* 1996). Recent studies have shown that there is a proteinaceous mechanism of biofilm formation (Figure 1.3.b.) which is independent of *ica* or PIA (Fitzpatrick *et al.* 2005;Hennig *et al.* 2007;Rohde *et al.* 2005).

PIA is a homoglycan composed of β-1, 6-linked *N*-acetylglucosamine residues containing 15 to 20% de-*N*-acetylated amino groups and positively charged (Figure 1.4.). These positive charges have been shown to be of major biological importance in biofilm formation, virulence, and immune evasion (Vuong *et al.* 2004b).

The *icaADBC* operon was identified by Tn917 transposon mutagenesis of *S. epidermidis* and screening for strains deficient in biofilm formation. This operon comprises 4 genes: *icaA, icaD, icaB,* and *icaC* (Figure 1.5.). The *icaA* gene product is a transmembrane protein which has *N*-acetylglucosaminyltransferase activity with UDP-*N*-acetylglucosamine as a substrate. IcaA adds *N*-acetylglucosamine from UDP-*N*-acetylglucosamine to the growing PIA chain, requiring the *icaD* gene product for optimal activity. *N*-acetylglucosamine oligomers produced by IcaAD reach a maximal length of

20 residues. Only when *icaAD* is coexpressed with *icaC*, longer oligomer chains are synthesized. The *icaC* gene product is a putative membrane protein, which is hypothetically involved in externalisation and elongation of the growing polysaccharide and is essential for the synthesis of full-length PIA. The *icaB* gene product is a surface-attached protein responsible for deacetylation of the poly-*N*-acetylglucosamine molecule. Non-deacetylated poly-acetylglucosamine in an isogenic *icaB* mutant strain, most likely due to the loss of its cationic character cannot attach to the bacterial cell surface or mediate biofilm development.

The divergently transcribed *icaR* gene, which is located upstream of the *icaA* gene, encodes a transcriptional repressor which appears to be a member of the TetR family of transcriptional regulators and negatively regulates *icaADBC* expression.

In cases of PIA-independent biofilm formation, adhesive proteins have been suggested to be involved in the accumulation phase. In some strains (*ica*-positive or negative), biofilm formation is mediated by specific surface proteins such as accumulation-associated protein (Aap), biofilm-associated protein (Bap), or Bap homologue (Bhp) (Cucarella *et al.* 2001;Fitzpatrick *et al.* 2005;Hennig *et al.* 2007;Hussain *et al.* 1997;Lasa and Penades 2006;Tormo *et al.* 2005a).

1.5.3. Biofilm detachment

Once a mature biofilm has been established, cells or cell aggregates may be continuously released into the flowing extracellular fluid (bloodstream) on a chronic basis, with consequent bacteraemia as well as seeding to distant sites. In contrast to primary attachment and accumulation, detachment is poorly understood in *Staphylococcus* spp. However, several factors have been proposed to be involved in biofilm detachment including; mechanical forces (flow effect), changes in nutrient concentration, cessation of production of biofilm building material, and production of detachment factors which is mainly controlled by quorum-sensing (QS) system accessory-gene regulator (*agr*) (Otto 2008a;Otto 2009;Yao *et al.* 2005a). The detachment factors have been proposed to function via two detachment mechanisms (Figure 1.2.); enzymatic degradation of biofilm exopolymers (Boles and Horswill 2008) and disruption of non-covalent interactions by detergent-like molecules (Vuong *et al.* 2004a). Examples

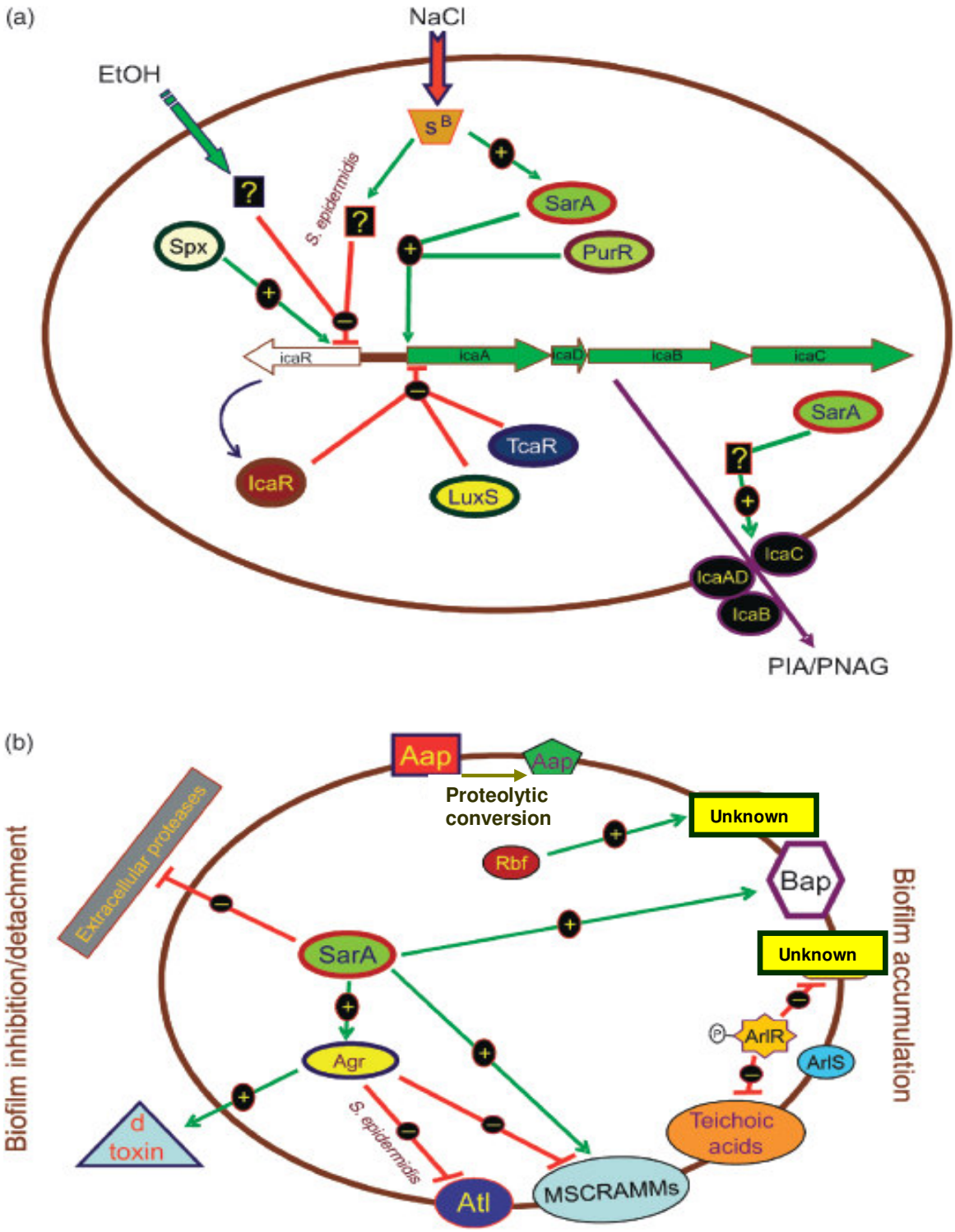


Figure 1.3. Model of *ica*-dependent (a) and *ica*-independent (b) biofilm mechanisms in *Staphylococcus* spp. (O'Gara 2007). The green and red arrows indicate positive and negative regulatory pathways, respectively.

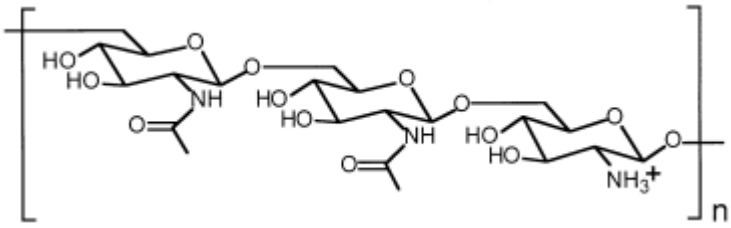


Figure 1.4. Structure of polysaccharide intercellular adhesin (PIA), a linear homoglycan composed of β-1, 6-linked *N*-acetylglucosamine residues (Gotz 2002).

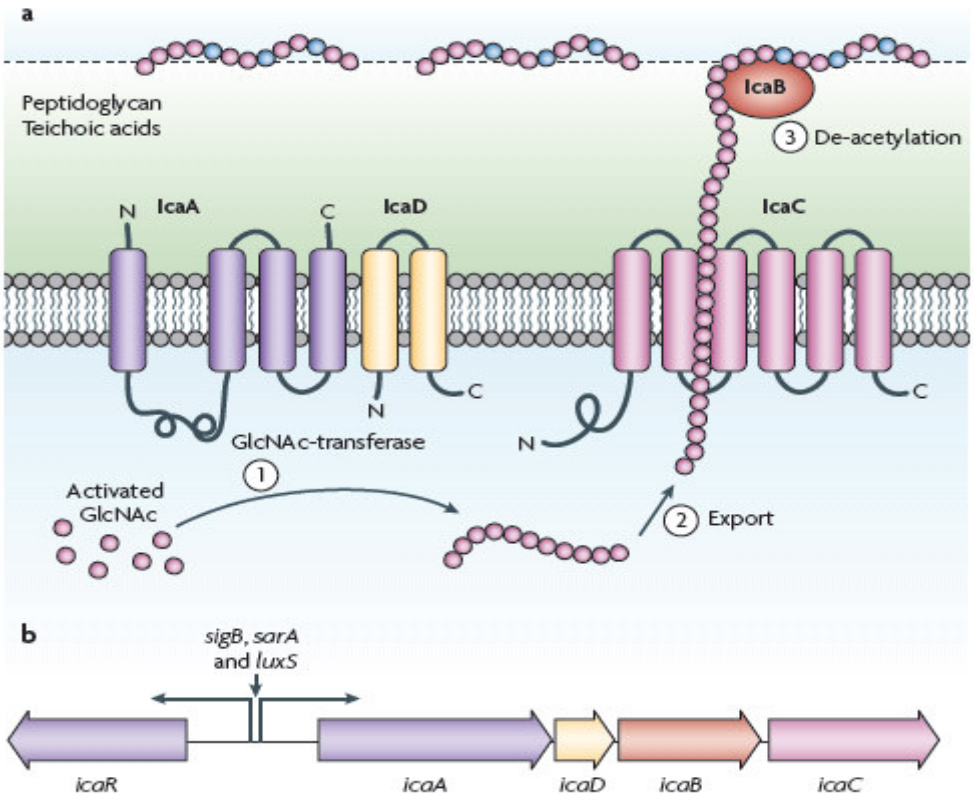


Figure 1.5. Schematic overview of PIA synthesis (a) and the gene arrangement in the *ica* operon (b) (Otto 2009).

of such factors in *S. epidermidis* are the short amphipathic phenol-soluble modulins (PSMs) and delta-toxin. In *Pseudomonas aeruginosa* a degrading enzyme (phosphatase) is encoded by the genetic locus that encodes the phosphate kinase responsible for the

production of the biofilm factor polyphosphate (Rashid *et al.* 2000). The same locus in *S. epidermidis* shows the same organization. However, the *ica* locus does not encode a protein to which a PIA-degrading activity could be attributed by similarity.

1.6. Regulation of biofilm formation

S. epidermidis possesses several regulator systems including *agr*, *luxS*, staphylococcal accessory regulator *sarA*, *sae*, and the alternative sigma factor σ^B which regulate biofilm formation in response to environmental conditions (Figure 1.6.). Until recently, many researchers have equated the staphylococcal biofilm matrix with PIA, which as we now know is not completely valid. Hence, the regulation of PIA expression is the best studied among the regulatory influences on staphylococcal biofilm formation. The regulators, for which a mechanism for their influence on biofilm formation has been described, are discussed below.

1.6.1. Quorum sensing systems

The QS systems are global regulators that control gene expression in response to cell density by small signaling molecules. The signal molecules of QS systems are small molecules called autoinducers (AIs). So far, three different types of AIs have been described: *N*-acylhomoserine lactones (HSL, AI-1) produced by Gram-negative bacteria, LuxS/autoinducer-2 (AI-2) common to Gram-positive as well as Gram-negative organisms, and oligopeptides produced from pre-proteins occurring in many Gram-positive bacterial species (Mack *et al.* 2007). The accumulation of high concentrations of AIs due to the increase in cell population density activates QS systems which in turn regulate the expression of various genes. In *S. epidermidis* two QS systems have been described to date: the *agr* system (Kong *et al.* 2006) and *luxS*/AI-2 system (Li *et al.* 2008;Xu *et al.* 2006). Both QS systems appear to repress the formation of biofilm.

1.6.1.1. agr

The *agr* locus consists of two divergent promoters, P2 and P3, the P2 transcript covers 4 genes (*agrA, agrC, agrD* and *agrB*) that are co-transcribed (Figure 1.7.). The

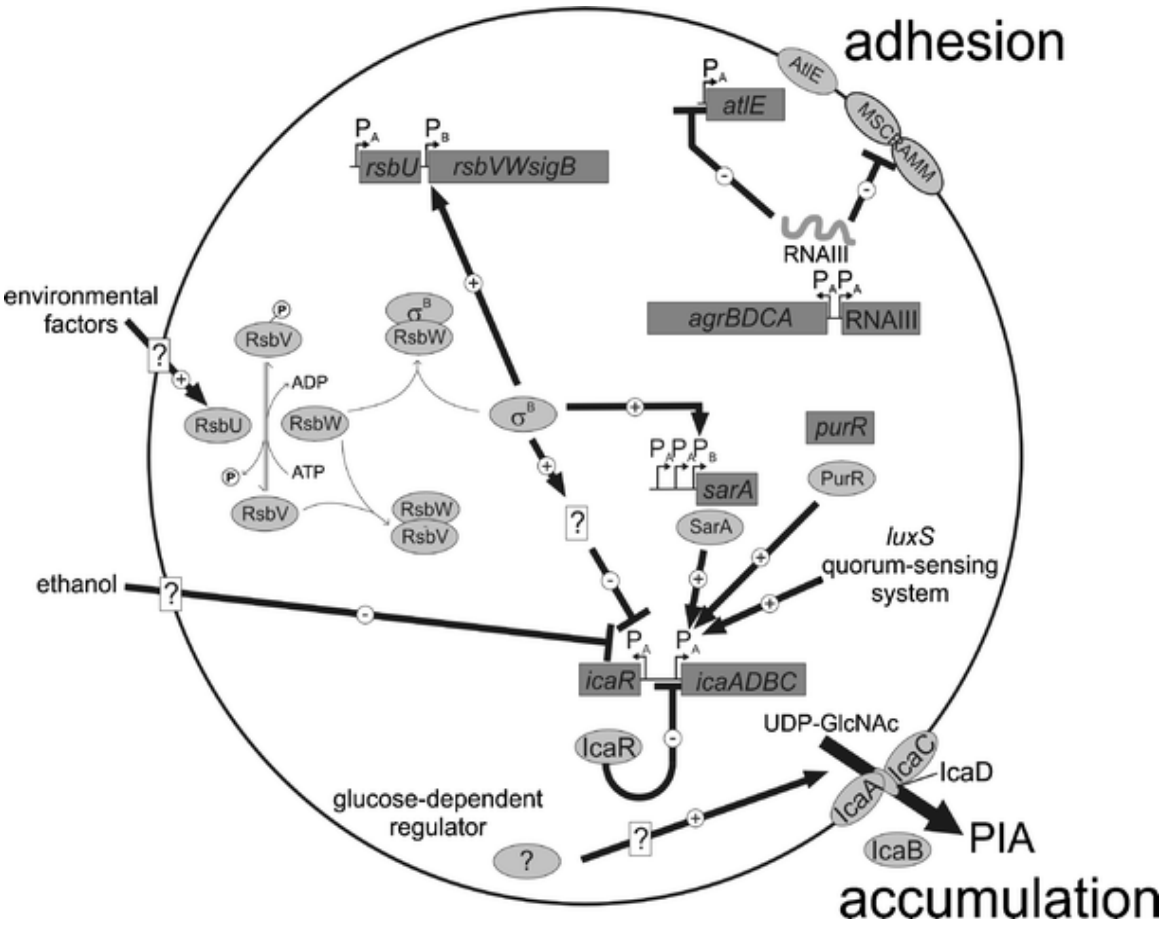


Figure 1.6. Schematic overview of the regulation of biofilm formation in *S. epidermidis* (Mack *et al.* 2007). Rectangles represent genes, ovals represent proteins.

agrB and *agrD* gene products are engaged in the production of the AI which is a short peptide (7 to 9 amino acids in length). The AI is encoded within the *agrD* gene as a larger polypeptide, and subsequently trimmed by the *agrB* gene product to form a thiolactone-containing ring structure. The *agrA* and *agrC* gene products constitute a classical two-component signaling transduction system. At the threshold concentration of the autoinducer peptide (AIP), the AIP is bound by AgrC, which is a membrane-bound receptor. AgrC phosphorylates AgrA and activates it. AgrA activates the P2 and P3 promoters. The P2 promoter controls expression of *agrB, agrD, agrC* and *agrA*, thereby closing the quorum sensing circuit, whereas activation of P3 promoter drives expression of the effector molecule RNAIII, which in turn controls transcription of the target genes

by a mechanism of which the details are still unknown. One possibility is that RNAIII acts via trans-acting factors, as RNAIII is potentially capable of forming a stable secondary structure that may create protein binding sites. RNAIII expression upregulates the expression of exoproteins including PSMs which play a role in detachment, and downregulates expression of surface proteins such as AtlE and other MSCRAMMs which play roles in attachment and binding to host matrix proteins.

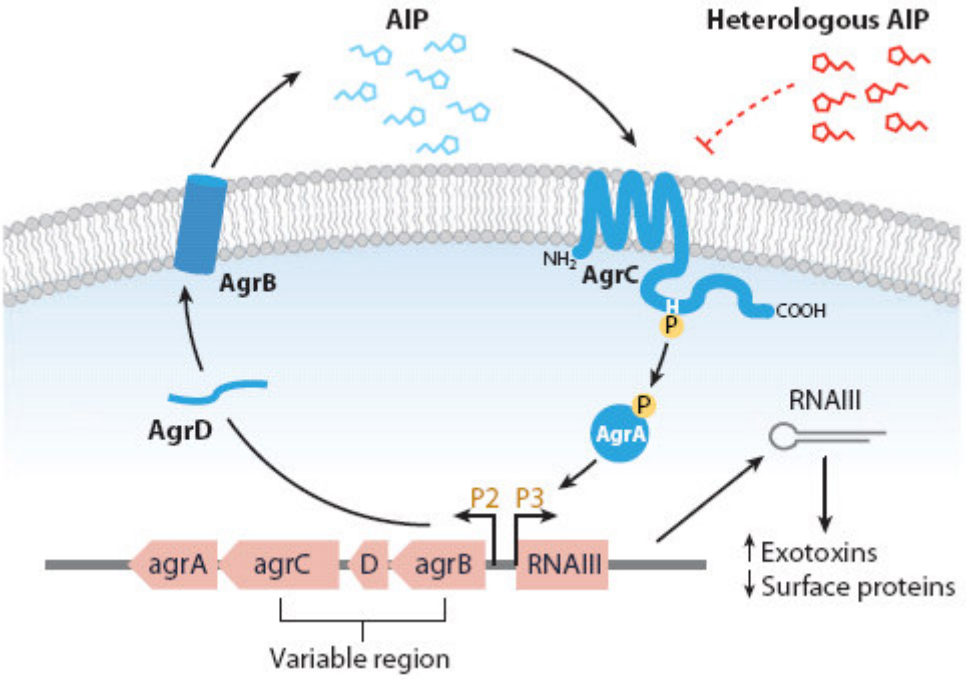


Figure 1.7. Schematic overview of *agr* QS system in *S. epidermidis* (Novick and Geisinger 2008).

In a biofilm, *agr* expression is limited to bacteria in the surface-exposed area and probably regulates biofilm detachment by upregulation of PSM effector molecules. Furthermore, *agr* mutants occur naturally in deeper layers of biofilm at a relatively high rate.

Due to sequence variation in the sequence of *agr* genes (*agrB*, *C*, *D*) AIs from different *S. aureus* and *S. epidermidis* strains can be divided into 4 different classes named I to IV, in which AIP produced by one group inhibits *agr* expression in other

groups (Otto *et al.* 2001). Because of this cross-inhibition phenomenon, it is speculated that staphylococcal biofilms are single species or even single strain biofilms instead of mixed-strain biofilms and the *agr* pheromones and system have been suggested as a target for the development of anti-staphylococcal therapeutics (Balaban *et al.* 2003;Balaban *et al.* 2007). However, recent studies demonstrated that *agr* dysfunction or blocking of the quorum-sensing increased biofilm formation and persistence of an infection (Otto 2004a;Vuong *et al.* 2000).

1.6.1.2. luxS

The other QS system in *S. epidermidis* that has a significant impact on biofilm development is the *luxS*/AI-2 system. AI-2 which is supposed to be designed for interspecies communication is synthesized by the *luxS* gene. AI-2 is mainly expressed during the logarithmic growth phase and its expression is reduced in the stationary growth phase in *S. epidermidis*. It has been demonstrated that AI-2 via a signaling function, influences biofilm formation *in vitro* and enhances virulence in a rat model of biofilm-associated infection. This is most likely due to regulation of *ica* operon genes and PIA production and its control of expression of PSMs. However, in contrast to the *agr* system, the details of the mechanism of the AI-2 signaling function and the sensors or transporters for AI-2 in *S. epidermidis* are not yet known. The regulatory effect of *luxS* on expression of *ica* operon genes couldn't be confirmed in another study by the same research group (Li *et al.* 2008).

1.6.2. Alternative sigma factor B

Bacterial RNA polymerase enzymes require a specialized subunit called sigma factor (σ) to recognize and contact their promoter. Three sigma factors (σ^A, σ^B, σ^H) have been identified in *Staphylococcus* spp., among which σ^B has been intensively studied (Kies *et al.* 2001). In *S. epidermidis* the σ^B operon consists of four genes, *rsbU, rsbV*, *rsbW* and *sigB*, among which *rsbU* is preceded by a SigA-dependent promoter, whereas a SigB-dependent promoter precedes the last three genes. SigB increases its own transcription as a consequence of its activation, further inducing the entire σ^B regulon.

Activity of sigma factors is increased in response to specific signals or stress conditions (Figure 1.6.).

SigB activity is regulated by a regulatory cascade including the anti-sigma factor RsbW (negative regulator), the anti-anti-sigma factor RsbV and the RsbV-specific phosphatase RsbU (positive regulators). RsbW is the key negative regulator of SigB and inactivates it by binding to it. RsbV, in its dephosphorylated form, modulated by RsbU under stress conditions via specific signals can bind competitively to RsbW, resulting in release of free and active SigB (Knobloch *et al.* 2001).

The influence of σ^B on biofilm formation appears to occur via repression of *icaR* transcription, which in turn represses transcription of *icaADBC* (Knobloch *et al.* 2004). However, it has been recently demonstrated that σ^B is less important for biofilms under high-oxygen conditions than under low-oxygen conditions and the regulation of *ica* operon expression and biofilm development in *S. epidermidis* and *S. aureus* is different. Unlike in *S. epidermidis*, mutations in the *S. aureus sigB* locus do not result in a biofilm negative phenotype (Cotter *et al.* 2009).

1.6.3. *sarA*

The staphylococcal accessory regulator locus *sarA* encodes a DNA-binding protein that is involved in the regulation of the biofilm formation in *S. epidermidis* by affecting *ica* operon transcription in an IcaR-independent manner or by interfering with PIA production at a later stage (Figure 1.3. & 1.6.) (Tao *et al.* 2006;Tormo *et al.* 2005b). The regulatory pathways influencing biofilm formation dependent on SarA and SigB are different, although these pathways are interconnected as one of the three *sarA* promoters is SigB-dependent.

1.6.4. Other regulators

Several other regulatory gene loci, which influence the biofilm formation process in *S. epidermidis* have been identified by transposon Tn917 insertional mutagenesis or phase variation of biofilm formation (Conlon *et al.* 2004;Heilmann *et al.* 1996;Heilmann and Gotz 1998) but their mechanism of action and their functions are still unknown.

1.6.5. Environmental factors

It has been shown that *S. epidermidis* biofilm formation *in vitro* can be influenced by environmental factors such as, oxygen (O_2), glucose, iron (Fe), salt, ethanol, antibiotic stress, Mg^{2+}, temperature and EDTA which may act through regulatory proteins like Agr, σ^B and the intercellular adhesion regulator, IcaR.

The *icaADBC* operon is activated in a SigB-independent, IcaR-dependent manner by ethanol (Knobloch *et al.* 2001). Induction of biofilm formation by addition of glucose is IcaR-, *icaADBC*-independent and is suggested to depend on the continuous translation of a so far unidentified glucose-regulated protein (Conlon *et al.* 2004;Dobinsky *et al.* 2003). The *icaADBC* operon is activated in a SigmaB-, IcaR-independent manner by sodium chloride (NaCl) (Conlon *et al.* 2004). In *S. aureus*, the *rbf* (regulator of biofilm formation) gene, regulates biofilm development in media supplemented with glucose or NaCl (Lim *et al.* 2004).

1.7. Prevention and treatment of *S. epidermidis* biofilm-associated infections

Currently, the only completely effective method for curing biofilm infections is to remove the infected device, a risky, costly and stressful procedure. Biofilms act as a shield that protects the bacterial cells from harsh environmental conditions such as UV exposure, metal toxicity, acid exposure, dehydration and salinity, phagocytosis and antibiotics or other antimicrobial agents (Hall-Stoodley *et al.* 2004). The central problem with microbial biofilm infections is their propensity to resist clearance by the host immune system and all antimicrobial agents tested to date. In fact, compared to their free floating (planktonic) counterparts, bacteria within a biofilm (sessile) are up to a 1000 times more resistant to antimicrobial agents (Stewart and Costerton 2001). Factors that contribute to the general resistance of biofilms are;

- Restricted penetration of antimicrobial compounds.
- Decreased growth rate of bacteria in a biofilm making them less susceptible to antimicrobials targeting metabolic pathways.

- A distinct phenotype that confers resistance to antimicrobials and the host immune defence and facilitates horizontal gene transfer.
- The presence of persister cells. Persisters are a unique class of inactive but highly protected cells that withstand a wide range of antimicrobial agents.

Different strategies, used to combat the biofilm infections have been summarized in table 1.1. The preventive strategies target functional molecules, gene systems and regulatory circuits, which control early stages of biofilm development, before establishment of a mature biofilm. Therapeutic strategies are aimed at the disintegration of established biofilms and include QS perturbation, which leads to the downregulation of molecules stabilising the biofilm architecture, or the use of enzymes to dissolve the biofilm matrix (Speziale *et al.* 2008).

Another frequently utilized option involves modification of biomaterial surface to overcome bacterial colonization and development of biomaterial capable of exerting antimicrobial action. This strategy is based on the prophylactic use of antibiotics and includes immersion, coating and matrix loading, preventing microbial contamination from occurring on the surface of the medical device in the first instance and therefore preventing bacterial colonisation and biofilm formation. The problem with this method is the selection of resistance.

Immunoprophylaxis and immunotherapy targeting proteins and surface components that are expressed *in vivo* and are important for biofilm formation are promising new approaches to prevention and treatment of biofilms. Several recent studies have shown that application of antibodies against surface components of *S. epidermidis* can affect the rate of biofilm formation or adherence of bacteria to the medical devices *in vitro*. Cerca *et al.* showed that antibodies against PIA readily penetrated the biofilm and bound to the sessile cells (Cerca *et al.* 2006), although sessile bacteria exhibited more resistance to opsonic killing than their planktonic counterparts. Using polyclonal antibodies against a Fg-binding protein from *S. epidermidis* (Fbe), Pei *et al.* could block adherence of *S. epidermidis* to Fg-coated catheters *in vitro* (Pei and Flock 2001a). Sun *et al.* showed that monoclonal antibodies against Aap can significantly reduce the accumulation phase of biofilm formation by *S. epidermidis in vitro* (Sun *et al.* 2005). Maira-Litran *et al.* showed that vaccination of rats with purified PIA/PNAG can elicit

Table 1.1. Novel antibiofilm approaches (Mccann *et al.* 2008).

Approach	Mechanism of action	Target
QS interference		
RNA III-inhibiting peptide (RIP)	QS interruption	RNAIII synthesis
Impairing adhesion		
Biosurfactants, including RC14 biosurfactant 'surlactin'	Anti-adhesive activity; interference with initial bacterial attachment	Microbial adhesion
Furanone compounds	Reducing adhesion and colonisation	Gene encoding adhesion and slime production?
Diterpenoids (salvipisone and aethiopinone)	Destabilising biofilm matrix allowing detachment +/ altering bacterial cell surface hydrophobicity	Biofilm matrix +/ bacterial cell surface
Targeting slime formation		
N-acetyl-D-glucosamine-1-phosphate acetyltransferase (GlmU) inhibitors (N-substituted maleimides)	Inhibiting bacterial cell wall synthesis and PIA formation	PIA biosynthetic enzymes; GlmU enzyme
N-acetylcysteine (NAC)	Reducing production of extracellular polysaccharide matrix and promoting disruption of mature biofilm	Extracellular polymeric matrix
Bacteriophage therapy; phage K & bacteriophage 456	Lytic activity on biofilm cells	Biofilm exopolysaccharide and biofilm cells
Immunotherapy		
FN binding receptor monoclonal antibodies (MAbs)	Blocking adhesion	FN binding receptor
Anti-PIA antibodies	Inhibition of PIA formation	PIA
Surface binding protein/Fbe antibodies	Blocking adhesion	Fbe
Anti-Aap domain B antiserum Aap antibodies	Inhibiting accumulation and intercellular adhesion	Aap
Enzymatic removal		
Oxidoreductases & polysaccharide-hydrolysing enzymes	Enzymatic removal and disinfection of biofilm	Biofilm matrix
Lysostaphin (staphylolytic endopeptidase)	Disruption of biofilm matrix and killing of released bacteria	Peptidoglycan pentaglycine interpeptide cross-bridges of staphylococcal cell wall
Dispersin B (DspB)	Enzymatic degradation of cell bound exopolysaccharide adhesin, an essential component of the biofilm polymeric matrix	β-1,*N*-acetyl-D-glucosamine
Serratiopeptidase	Induces biofilm degradation via proteolytic activity, also enhances antibiotic activity	Biofilm slime matrix
Immunomodulation		
Interferon γ	Reversal of macrophage deactivation in the vicinity of implanted biomaterial	Macrophages

protective immunity against both CNS and *S. aureus* (Maira-Litran *et al.* 2004). However, recent studies have shown the existence of PIA-independent biofilm mechanisms in both *S. epidermidis* and *S. aureus*.

A list of surface-expressed components of *S. epidermidis* as the potential targets for vaccine development including PIA, teichoic acids, proteinaceous adhesins and cell-wall anchored proteins has been summarized by Götz (Gotz 2004). In a recent publication, Otto reviewed the potential use of surface components of *Staphylococcus* spp., with emphasis on MSCRAMMs as target for developing an anti-*Staphylococcus* spp. vaccine (Otto 2008b).

Currently, most vaccines available for human use are whole (killed or attenuated) microorganisms or subunit vaccines, developed using conventional methods that follow the paradigm established by Pasteur over a century ago. However, *S. epidermidis* is a ubiquitous colonizer of human skin, and staphylococcal infections don't cause immune protection in the long term. Accordingly, whole-cell vaccines generally do not achieve protection from *S. epidermidis*.

In 2 different independent studies, immunogenic cell wall-associated proteins of *S. epidermidis* were experimentally identified (Pourmand *et al.* 2006;Sellman *et al.* 2005). However, the potential use of these targets for vaccination or immunotherapy against *S. epidermidis* biofilms in FBI has not been investigated.

Conventional vaccinology is time-consuming and inadequate in the development of vaccines for those pathogens that are antigenically diverse or cannot be cultivated in the laboratory. Recently, there have been two revolutions in vaccine design; i) use of modern recombinant DNA technology to produce subunit vaccines, ii) using whole-genome sequences and bioinformatics termed 'reverse vaccinology' (Mora *et al.* 2003) to identify potential vaccine targets. Reverse vaccinology doesn't have the limitations of conventional vaccinology, and vaccine candidates can be rapidly identified *in silico* before being subjected to confirmatory studies. However, non-protein antigens (e.g. polysaccharides, lipopolysaccharides and glycolipids) can't be studied using this method.

The first step in reverse vaccinology is identification of the complete repertoire of antigens that are expressed on the surface. The identification of potential antigens can be done using 3 different approaches; i) proteomics (Sellman *et al.* 2005), ii) immunomics

(Pourmand *et al.* 2006), iii) *in silico* analysis (this study), followed by high-throughput expression and screening of vaccine candidates. Vaccine development proceeds by epitope mapping and quantification and qualification of immune response to the used epitopes.

An advantage of immunotherapeutic methods is the lesser risk for the development of resistance. However, S. *epidermidis* has certain features including the occurrence of allelic variation, redundant protein functions or altered stress-induced expression properties that make the development of a vaccine difficult. *S. epidermidis* is also a common part of the human microflora and eradication of *S. epidermidis* may lead to severe disturbances of the physiological balance on human epithelia and allow potentially more harmful microorganisms to take its place. In addition, animal protection models, in contrast to humans, have only limited value for anti-staphylococcal vaccine development. Thus, only at the stage of clinical trials can the efficacy of an anti-staphylococcal vaccine be evaluated, as two high-profile vaccine preparations have failed clinical trials within the last few years (Otto 2008b).

1.8. Aims and general outline of this work

The aims of this work were as follows:

1. To identify potential target(s) for vaccines against *S. epidermidis* biofilms.
2. To establish the protective effect of antibodies against the selected target(s) in prevention and treatment of FBI in '*in vitro*' and '*in vivo*' models.
3. To establish the protective effect of immunization with the selected target(s) on '*in vivo*' FBI.
4. To investigate the role(s) of the selected target(s) in *S. epidermidis* biofilm formation.

The general outline of this work is as follows:

First, genes coding for *S. epidermidis* 'surface-exposed proteins' [Ses; terminology used previously by Gill *et al.* and Bowden *et al.* (Bowden *et al.* 2005;Gill *et*

al. 2005)] were identified by an *in silico* procedure. From these, 13 proteins were selected based on protein size, number of antigenic determinants and the role in *S. epidermidis* biofilm formation and pathogenesis of the protein family to which the candidate protein belongs.

The presence of these 13 genes in *S. epidermidis* clinical isolates was investigated and - using previously described *in vitro* and *in vivo* rat models for FBI (Vandecasteele *et al.* 2001;Vandecasteele *et al.* 2002) - the expression of these 13 genes was investigated in planktonic and sessile bacteria during FBI.

For the biofilm inhibition studies, extracellular coding regions of 5 out of the 13 genes were cloned, recombinantly expressed and purified. Rabbit hyperimmune sera against these 5 recombinant proteins were raised and the total IgG fraction from pre- and hyperimmune sera was isolated. The effect of pre- and post-immune IgGs on primary attachment and accumulation phases of *S. epidermidis* biofilm formation was tested *in vitro* (Christensen *et al.* 1985;Sun *et al.* 2005).

Finally, SesC, against which antibodies exhibited the highest inhibition of biofilm formation *in vitro* was selected for further investigation. The effect of different concentrations of purified specific anti-SesC IgGs (αSesC-IgGs) was tested *in vitro* on primary attachment, overnight biofilm formation and established (1-day old) biofilms of different *S. epidermidis* strains. We also tested the effect of αSesC-IgGs on established biofilms of *S. epidermidis* in our *in vivo* rat model for FBI. Finally, rats were immunized with SesC and the effect of immunization on FBI was evaluated.

To unravel the structure and function of SesC, different bioinformatics tools were used and we studied the effect of expression of *sesC* in the *sesC*-negative strain *S. aureus* RN4220 (Kreiswirth *et al.* 1983).

CHAPTER 2:

IDENTIFICATION OF SES PROTEINS OF *S. EPIDERMIDIS*

2. Identification of Ses proteins of *S. epidermidis*

2.1. Introduction

The first step in reverse vaccinology is *in silico* selection of potential antigens for vaccine development. The success of reverse vaccinology-based strategies is dependent on the criteria used in this *in silico* procedure. The surface components, in particular those surface proteins which are highly expressed in the blood stream and in biofilms, with a possible role in biofilm formation, are hypothesized to be the best antigens for development of a vaccine against biofilms (Otto 2008b).

Out of 57 identified Ses proteins using the *in silico* procedure, 13 proteins were selected for further investigation, based on the protein size, the number of antigenic determinants and the importance of the protein family, to which the candidate protein belongs, in *S. epidermidis* biofilm formation and pathogenesis.

2.2. Materials and methods

2.2.1. *In silico* selection of Ses proteins

The complete sequence of *S. epidermidis* ATCC 12228 (Zhang *et al.* 2003) was retrieved from the National Centre of Biotechnology Information (NCBI) GenBank (http://www.ncbi.nlm.nih.gov/Genbank/). We predicted the presence of a signal peptide at the N-terminus, using the SignalP server (http://www.cbs.dtu.dk/services/SignalP-2.0/). Thereafter, we predicted the number of transmembrane (TM) domains in proteins with a signal peptide, using the TMHMM server (http://www.cbs.dtu.dk/services/TMHMM-2.0/). Finally, we performed a retention domain prediction on the proteins with none or a single transmembrane domain, searching for lipobox motifs, peptidoglycan binding domains (PBD), choline binding domains (CBD) and LPXTG motifs. Proteins with two transmembrane domains were analyzed for LPXTG motifs as well. Retention domains were predicted using the PATTINPROT server (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_uploadfile.html). The prediction of protein

subcellular localization was reanalysed using the online tool PSORTb V.2.0.4 (http://www.psort.org/psortb/). The sequences of all identified Ses proteins were subjected to antigenicity analysis using the 'Predicting Antigenic Peptides' server (http://imed.med.ucm.es/Tools/antigenic.pl).

2.2.2. Gene identification

The sequences of selected *ses* genes were retrieved via the NCBI GenBank from the complete genome of the non-biofilm forming *S. epidermidis* strain ATCC 12228. All selected genes were subsequently sequenced in *S. epidermidis* strain 10b (Van Wijngaerden *et al.* 1999). The 10b strain is a strong biofilm-forming *S. epidermidis*, which was isolated from a patient with a proven CRI. On the basis of these sequences, all primers and probes were designed with Primer Express 2.0 software (Applied Biosystems Division of Perkin-Elmer) and were purchased from Eurogentec (Seraing, Belgium). In this study all fragments were PCR amplified using genomic DNA (gDNA) isolated from *S. epidermidis* strain 10b, unless otherwise specified.

2.2.3. Bacterial isolates and species identification

A total of 76 *S. epidermidis* clinical and commensal isolates from hospitalised patients (n = 60), skin of healthy individuals (n = 11) in our department and five previously described strains {10b, 1457 (Mack *et al.* 1992), ATCC 35984 [RP62A (Gill *et al.* 2005)], ATCC 12228 and TU3298 (Allgaier *et al.* 1985)} were collected. Clinical isolates were recovered from blood cultures of neonates (n = 45) with late-onset sepsis and an intravascular catheter in place at the NICU of the Erasmus MC–Sophia Children's Hospital in Rotterdam, (the Netherlands) and from different clinical specimens from patients (n = 15) hospitalized at the University Hospital Gasthuisberg (Katholieke Universiteit Leuven) in Leuven, Belgium. Species identification was with VITEK 2 (bioMérieux).

2.2.4. PCR screening of *ses* genes in clinical and commensal isolates

We performed a duplex PCR, amplifying both a *ses* gene and the 16S ribosomal RNA (16S rRNA) gene. The primers used for amplification of the *ses* genes are listed in

table 2.1., and primers for the 16S ribosome gene were previously described (Vandecasteele *et al.* 2001). For each strain, genomic DNA was extracted using a QIAamp DNA mini kit (Qiagen).

Table 2.1. List of primers used in PCR screening of *ses* genes in clinical and commensal isolates and for cloning in the pGEM-T Easy vector used as standard in real time PCR (Chapter 3.2.2.).

Locus Name	Forward primer 5'… 3'	Reverse primer 5'… 3'	Fragment length
SE0193	CCCGTAGCTAAGAAAATCGC	GGCTTGGTTCTTCCGCATTC	469
SE0331	CCTCATCAGTCGAATCCTC	CAGCATCATGTGCTTTAAT	477
SE0684	CTGCCGATACACCGGATGG	GCGTCGTTTCAGGCCCTAC	579
SE0828	TCAACCATCAGCACCTGGAA	TGATCACCAGTGGCACCTTG	423
SE1106	TGGGCCACTCAATACAGTCA	TTGGCGTGTTTCTGTCTTTG	435
SE1500	CTGGGTTGACCAAACTTGGC	GGTTTTTCTCCAATAAAGCCA	378
SE1501	CCAATTACTAGTATTAAATTCAG	CTACACTGTTAGACGTGAG	355
SE1588	GGTACTACGGTTGAAGAGGG	TTGTGGTTCTTTATCGTCTTTAG	390
SE1981	CAGGTGCCTTGGAATCGC	GCGTACCTTGCCAGTAGTC	441
SE2152	GCTATGAAAAATAGTGGTGGC	CGTAGTATGAATTGAGCTCAC	298
SE2212	GAAAGCCAAACAACAAAGTGA	CCACTTCACATCTTGAACTTG	325
SE2232	GTTGATAACCGTCAACAAGG	CATGTTGATCTTTTGAATCCC	388
SE2395	GACAGCATAAAAGAGGGAG	CCGTCACTTCGTAAACTTTCAC	499

2.3. Results

2.3.1. Selection of Ses proteins

Figure 2.1. shows a diagram of our *in silico* selection procedure. In total, 57 proteins have been identified as Ses proteins of which 32 were annotated as (conserved) hypothetical (data not shown). Seven LPXTG-containing proteins and 6 lipobox-containing proteins were selected for further investigation (Table 2.2.). Out of 13 selected proteins, 5 proteins of which the function had not yet been characterized, were selected to investigate their potential use as a target for biofilm inhibition (marked by * in table 2.2.).

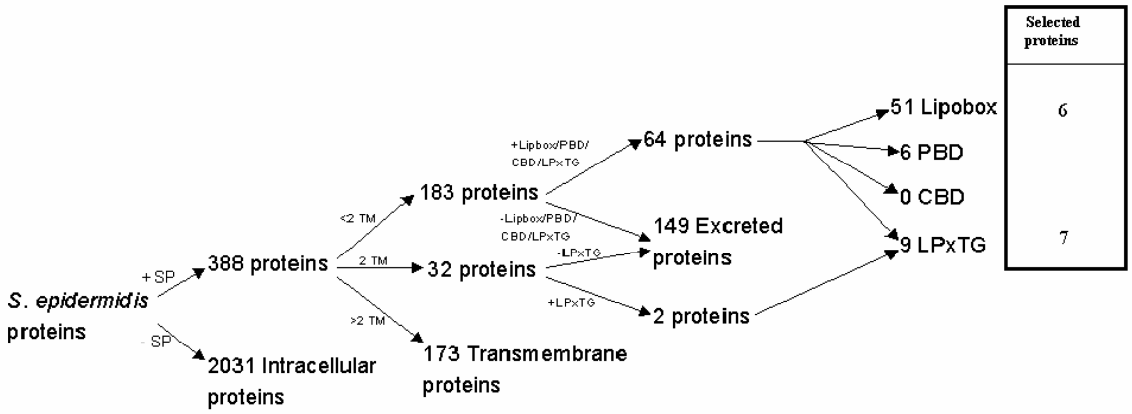


Figure 2.1. *In silico* selection of genes coding for Ses proteins of *S. epidermidis.*

2.3.2. Presence of *ses* genes in clinical and commensal isolates

Presence of *ses* genes in clinical and commensal isolates of *S. epidermidis* is listed in table 2.3. There was no significant difference for the prevalence of genes in clinical or commensal isolates.

2.4. Discussion

Four major types of cell surface proteins in Gram-positive bacteria are: (i) transmembrane proteins, (ii) lipoproteins, (iii) LPXTG-like proteins and (iv) cell wall binding proteins (Desvaux *et al.* 2006;Scott and Barnett 2006). Five LPXTG motif containing proteins (Aap, Bhp, SdrF, SdrG and SesI) are known to play important roles in

Table 2.2. List of selected Ses proteins by *in silico* procedure used in this study. **No. of AD:** number of antigenic determinants, ***:** selected for biofilm inhibition studies, **aa:** amino acid

Locus Name	Putative product name	Protein accession number	Protein size (aa)	Motif	No. of AD
SE2395	Ser-Asp rich fibrinogen-binding,bone sialoprotein-binding protein (SdrF)	NP_765950.1	1633	LPXTG	50
SE0331	Ser-Asp rich fibrinogen-binding, bone sialoprotein-binding protein (SdrG)	NP_763886.1	1056	LPXTG	29
SE1500	hypothetical protein (SesJ)	NP_765055.1	1012	LPXTG	40
SE0828	lipoprotein VsaC (SesE)	NP_764383.1	827	LPXTG	31
SE2232 *	conserved hypothetical protein (SesC)	NP_765787.1	676	LPXTG	20
SE1106 *	ABC transporter _membrane spanning protein	NP_764661.1	564	Lipobox	16
SE0684	peptide binding protein OppA	NP_764239.1	547	Lipobox	19
SE1981 *	nickel ABC transporter nickel-binding protein	NP_765536.1	491	Lipobox	18
SE1501 *	hypothetical protein (SesK)	NP_765056.1	415	LPXTG	11
SE1588	conserved hypothetical protein	NP_765143.1	402	Lipobox	13
SE2212	conserved hypothetical protein	NP_765767.1	388	Lipobox	16
SE0193	Zn-binding lipoprotein adcA	NP_763748.1	331	Lipobox	11
SE2152 *	hypothetical protein (SesB)	NP_765707.1	196	LPXTG	7

the pathogenesis of *S. epidermidis* infections (Arrecubieta *et al.* 2007;Cucarella *et al.* 2001;Davis *et al.* 2001;Hussain *et al.* 1997;Soderquist 2007). In publicly available genomes of *S. epidermidis* strains RP62A and ATCC12228, respectively 11 and 10 genes encoding LPXTG proteins have been identified (Bowden *et al.* 2005) including those already mentioned. Except for the 5 LPXTG proteins mentioned above, the role of these LPXTG proteins has not been studied yet.

Table 2.3. List of the selected *ses* genes and their distribution among *S. epidermidis* isolates. The values show the number of isolates positive for the gene as a proportion of the total number of isolates tested.

Locus Name	Name used in other studies (Bowden *et al.* 2005;Gill *et al.* 2005)	Name used in this study	Presence in commensal isolates (n= 11)	Presence in clinical isolates (n= 65)
SE0193		*sesN*	11/11	63/65
SE0331	*sdrG*		11/11	65/65
SE0684		*sesO*	11/11	65/65
SE0828	*sesE*		11/11	65/65
SE1106		*sesL*	7/11	45/65
SE1500	*sesJ*		1/11	3/65
SE1501	*sesK*		1/11	6/65
SE1588		*sesP*	11/11	64/65
SE1981		*sesM*	11/11	65/65
SE2152	*sesB*		11/11	65/65
SE2212		*sesQ*	11/11	65/65
SE2232	*sesC*		11/11	65/65
SE2395	*sdrF*		8/11	57/65

Lipoproteins can be involved in a large variety of physiological functions, i.e. adhesion, transport, receptors, enzymes or virulence factors (Sutcliffe and Russell 1995). ABC transporter systems belong to a family of proteins which in Gram-positive bacteria performs important roles as substrate-binding proteins, in antibiotic resistance, in cell signaling, in protein export and folding, in sporulation and germination, in conjugation, in biofilm formation and various other functions (Sutcliffe and Harrington 2002).

Hence, based on protein length, conserved domains, and the number of antigenic determinants, 7 LPXTG-motif containing proteins and 6 proteins with the longest amino acid sequences and a high number of antigenic determinants were selected for further analysis. All of the selected proteins are predicted to be located on the cell surface of *S. epidermidis*. For some of these proteins, a tentative role in adherence to host or foreign bodies has already been proposed.

SE0193 (Zn-binding lipoprotein AdcA) is a homologue of a gene product in the *adcCBA* operon in *Streptococcus gordonii* that plays an important role in biofilm formation of this species (Mitrakul *et al.* 2005). Upregulation of this gene has been documented in *S. epidermidis* biofilms by microarray analysis (Yao *et al.* 2005a), but its precise role in *S. epidermidis* biofilm formation is still unknown.

SE0331 or Fbe (Ser-Asp rich Fg-binding, bone sialoprotein-binding protein or SdrG), is a major factor in adherence of *S. epidermidis* to Fg-coated surfaces (Pei and Flock 2001b). It was demonstrated that antibodies against Fbe can block adherence of *S. epidermidis* to Fg-coated surfaces (Pei and Flock 2001a).

SE0193, SE0684, SE1106, SE1981 proteins are members of the ABC transporter family. SE1500, SE1501, SE1588, SE1981, SE2152, SE2212 and SE2232 are hypothetical proteins for which no information is available so far that links them to biofilm formation. SE0331, SE0828, SE1500, SE1501, SE2152, SE2232, SE2395 belong to the LPXTG protein family.

SE0828 (lipoprotein VsaC) is a homologue of the VsaC protein of *Mycoplasma pulmonis* which is a variable surface lipoprotein antigen. Proteins encoded by this locus are important in strong adherence to polystyrene and erythrocytes and resistance to complement (Simmons *et al.* 2004).

SE2395 or SdrF (Ser-Asp rich Fg-binding, bone sialoprotein-binding protein), is a member of Sdr proteins. The SdrF, SdrG and SdrH proteins are cell-wall associated proteins that specifically bind host proteins and which all have a highly conserved motif of which the consensus sequence is TYTFTDYVD. Sdrf is a Cn-binding MSCRAMM (Arrecubieta *et al.* 2007).

2.5. Conclusion

The presence of proteins previously identified as surface proteins among the list of proteins identified as Ses proteins by our *in silico* procedure confirms the reliability of our method. However, further investigation is needed to validate the presence and expression of the selected proteins on the surface of *S. epidermidis*. The presence of some of the selected *ses* genes in all tested isolates might be linked to an essential function of the gene in *S. epidermidis*.

CHAPTER 3:

IN VITRO AND *IN VIVO* GENE EXPRESSION STUDIES

3. *In vitro* and *in vivo* gene expression studies

This chapter is based on the following reference: Mohammad Shahrooei, Vishal Hira, Rita Merckx, Benoit Stijlmans, Peter W.M. Hermans, Johan Van Eldere. 2009. Inhibition of *Staphylococcus epidermidis* biofilm formation by rabbit polyclonal antibodies against SesC protein. Infection and Immunity. 77(9):3670-8.

3.1 Introduction

After the *in silico* selection of potential vaccine antigens, the next step was to validate them by identification of their expression *in vitro* and *in vivo*. However, since biofilm cells show altered gene expression patterns compared to their planktonic counterparts (Vandecasteele *et al.* 2003;Vandecasteele *et al.* 2004;Yao *et al.* 2005b;Yao *et al.* 2005a), expression of the candidate genes in biofilm cells must specifically be investigated.

A technique for direct quantification of bacterial transcripts during *in vitro* and *in vivo* FBI based on quantitative PCR has been developed by Vandecasteele *et al*. (Vandecasteele *et al.* 2001;Vandecasteele *et al.* 2002). This methodology has been previously used to document the time-dependent induction *in vitro* and *in vivo* of structural biofilm genes, such as *ica* operon genes, *aap*, *mecA*, and 16S rRNA gene as a determinant of metabolic activity (Vandecasteele *et al.* 2003;Vandecasteele *et al.* 2004). Using this method, expression of iron-responsive genes such as *sirR* and the *sit* operon and global regulator genes such as *sarA*, *sigB*, and *agr* during the time course of biofilm development *in vitro* and *in vivo* was investigated (Massonet *et al.* 2006;Pintens *et al.* 2008).

3.2. Materials and methods

3.2.1. Bacterial strains and growth conditions

For all experiments, unless specifically mentioned, a previously described clinical *S. epidermidis* 10b strain was cultured in Brain Heart Infusion, (BHI, Oxoid).

3.2.2. Gene identification, cloning and quantification of copy number

The sequences of selected 13 *ses* genes (Table 2.2.) were retrieved from the complete genome of the non-biofilm-forming *S. epidermidis* strain ATCC 12228 (NC_004461) from NCBI GenBank. On the basis of these sequences, primers and probes were designed with Primer Express 2.0 software (Applied Biosystems Division of Perkin-Elmer) and were purchased from Eurogentec (Seraing, Belgium). All primers and probes used in gene expression studies were listed in table 2.1. and 3.1. All fragments were PCR amplified using gDNA isolated from *S. epidermidis* strain 10b. PCR was performed on a GeneAmp PCR System 9700 (PE Applied Biosystems).

The PCR-products of all genes amplified using primers listed in table 2.1. were cloned in the pGEM-T Easy vector system (Promega) according to the instructions of the manufacturer. Pure plasmids DNA were isolated using the High Pure Plasmid Isolation Kit (Roche Diagnostics), sequenced on the ABI 310 (PE Applied Biosystems) according to the manufacturer's instructions, and quantified with the Genequant RNA/DNA calculator (Amersham Pharmacia Biotech).

3.2.3. Model for gene expression in planktonic and sessile bacteria *in vitro*

For *in vitro* studies, 20 µl of a frozen culture of *S. epidermidis* 10b was inoculated in 5 ml fresh BHI and incubated at 37°C in a shaking incubator at 250 rpm. Bacteria were grown to the end-exponential growth phase, pelleted, and resuspended in 0.9% NaCl. Seven mm fragments of a commercial polyurethane intravenous catheter (Arrow International, Reading, Pa.) were added and the mixture was incubated at 37°C. After varying incubation periods (0, 10, 35, 60, 120, and 180 min), 16 samples, from 2 independent experiments, were taken for DNA and RNA extraction, from both planktonic and sessile bacteria.

3.2.4. Rat model for *in vivo* foreign body infection

For the *in vivo* rat model, first-generation descendants of inbred germfree Fisher rats were used. This rat strain was inbred and maintained under germ-free conditions in the Rega Institute, University of Leuven, since 1965 (Van Wijngaerden *et al.* 1999). The rats were exposed to normal rat flora from birth and were labeled ‘ex-germfree Fisher rats

(EGF)'. All animal experiments were conducted in compliance with the guidelines for animal experimentation.

Seven mm long catheter fragments were inoculated with a low inoculum of *S. epidermidis* 10b (10^4 cells/catheter) prior to implantation by incubation for 20 min at 37°C in a 0.9% NaCl suspension of *S. epidermidis* 10b. This resulted in a 100% infection rate. Anesthesia of rats was induced with inhalation of enflurane gas (alyrane™, Pharmacia). Rats were kept asleep during the implantation procedure by a mix of enflurane (20%) and oxygen (80%). The back of each rat was shaved over a large area; the skin was disinfected with 0.5% chlorhexidine in 70% alcohol and allowed to dry. A 10-mm incision was made at the base of the tail and the subcutis was dissected to create 3 subcutaneous tunnels. In each rat, eight catheter fragments were inserted at least 2 cm from the incision; the distance between two fragments was at least 1 cm.

For catheter explantation, rats were euthanized by CO_2 inhalation. The skin was disinfected before removal of the catheter fragments. All catheter fragments from the same animal were used for a single time point. In each experiment, baseline gene expression levels in sessile bacteria before implantation were determined (time zero; n = 16). A total of 176 polyurethane catheter segments were implanted and explanted at 11 different time points. The time points used were 15 min (n = 16), 1 h (n = 16), 2 h (n = 16), 4 h (n = 16), 6 h (n = 16), 12 h (n = 16), 24 h (n = 16), 2 days (n = 16), 4 days (n = 16), 7 days (n = 16), and 14 days (n = 16). Data for each *in vivo* time point were obtained from 16 independent measurements generated in 2 independent experiments. DNA and RNA isolation and cDNA synthesis were performed immediately after explantation.

3.2.5. DNA and RNA extraction and cDNA synthesis

DNA and RNA were extracted from planktonic and sessile bacteria grown *in vitro* and *in vivo* as previously described (Vandecasteele *et al.* 2001;Vandecasteele *et al.* 2002). Briefly, for bacteria in suspension (planktonic bacteria), an aliquot of the bacterial suspension with a maximum of 10^9 CFU (colony forming unit) was rapidly cooled on ice. The bacteria were pelleted, and the pellet was suspended in 500 µl of NAES buffer (50 mM sodium acetate [pH 5.1], 10 mM EDTA, 1% sodium dodecyl sulfate) and added to a FastRNA blue tube (Bio 101, Carlsbad, Calif.) with 500 µl acidified phenol-chloroform

(5:1; pH 4.5; Ambion, Austin, Tex.) at room temperature. For sessile bacteria, the colonized catheter fragment was washed with 1 ml of 0.9% NaCl and added directly to a FastRNA tube containing 500 µl of NAES buffer and 500 µl of acidified phenol-chloroform. The FastRNA tubes were shaken for 23 s at 6000 rpm with a FastPrep instrument (FP 120; Bio 101, Savant, Holbrook, N.Y). After shaking, the tubes were centrifuged.

After centrifugation, the supernatant was collected and precipitated with 520 µl of isopropyl alcohol and 35 µl of 3 M sodium acetate. The pellet was washed with 70% ethanol and resuspended in 150 µl of RNase-free water. Fifty microliters of this sample was diluted 1/10 and used for quantification of gDNA. The remaining 100 µl was purified with an RNeasy mini kit (Qiagen, Hilden, Germany) and treated with RNase-free DNase (Qiagen) on RNeasy columns according to the instructions of the manufacturer. The RNA was finally dissolved in 60 µl of RNase-free water. Reverse transcription of the RNA was performed with Moloney murine leukemia virus (MMLV) reverse transcriptase (Promega, Madison, Wisconsin, USA).

For RT-PCR we used 100 U MMLV reverse transcriptase (Promega, Madison, Wisconsin, USA) with the supplied buffer, 20 U RNasin (Promega), 100 µM random hexamers (Amersham Pharmacia Biotech), 1 mM of each dNTP, 9 µl RNA for planktonic samples and 36 µl for samples of sessile bacteria in a total volume of 60 µl. Reaction conditions were as follows: preheating RNA sample for 10 min at 70°C followed by addition of the reaction mix. cDNA was prepared from this mixture through incubation for 1 h at 42°C followed by heating at 99°C for 2 min for enzyme denaturation and rapid cooling to 4°C.

3.2.6. Taqman PCR

Gene quantification was performed on the ABI Prism 7700 Sequence detection System (PE Applied biosystems) using primers and probes labeled with the reporter dye 6-carboxy-fluorescein (6'-FAM) at the 5' end and with the quencher dye 6-carboxy-tetramethyl-rhodamine (TAMRA) at the 3' end (Table 3.1.). In brief, quantitative PCR was done with 2 µl of cDNA, 12.5 µl of 2X Taqman PCR master mix (PE Applied Biosystems), 900 nM of each primer, and 200 nM probe in a final volume of 25 µl.

Table 3.1. List of primers and probes used in real time PCR gene quantification.

***ses* gene**	**Forward primer 5'… 3'**	**Reverse primer 5'… 3'**	**Probe, labeled FAM-5' and 3'-TAMRA**
sesN	AGCACGGAGAAGGTCACGAA	GACTTACTACAGGATCCAACCAAATGT	AACATCATCACCATGGAAAATACGACCCA
sdrG	TCGATTAAACGTGTAACCGTAAATCA	TCACTATCATCATATCCTTCAGTAATACTTTGA	TAGCGGCGGAACAAGGTTCGAATGTTAA
sesO	GGGTGGAGTCCTGATTATC	TACTTGCGGATTCGTTAAG	CCAGTCGCTCCTGTTTATC
sesE	TCAAGCCGGTGCTACGGTTA	ACCAGTTGCCGTTTGATCGT	ATGGTCAAGACGGGAATCAA
sesL	TTGGGCAGATCATCTTGGTTATAC	GACTTACTACAGGATCCAACCAAATGT	CTGATTTCGGTGAAACTGAATACGGAGATAATACAA
sesJ	CACTTGGGTAGATGGTTCTAA	TTTTTCCGGTGTATAATTGTT	CTGCTTTGTTTTCTGGGAATGAA
sesK	CCCTAAAGATGAAACTACTGTTCATGAT	TGCTATCCATTCGAAACTTTCATTAT	ACAATGTGACAAAACAGGGTGTACTAAAAGTAAACGAA
sesP	TCAACTGCAGCTGATTATGA	GAGAGCACGTGGAGTATTTC	TACGGACAGGCAGAAACGATAGG
sesM	AACCTTATCCAGAATTAATGTCTGAACTC	CAGGTTGATCAGTTACTTTGTTTTTAGC	CAAGCCCATTTGCTGCTATTTACGACACAAA
sesB	GTGTATGCGGATGAAAATACT	CAGGGGTTTTAGCATCTTC	CTGCAAACCAAAATCAAGGAACA
sesQ	GGGGATTATTTCACAATTGA	CACTGCTTGTGTCTGTTGAG	AATAAATCTGGTGCAGGTGTCGG
sesC	AGCATCACCATCTAATAAAAACGAAA	CCATCATTACTTTTATCGTCTTTACTATCAC	TAACAAAGAAGAATCTAGTACGACAACAAATCAATCCGA
sdrF	ATTAGACCGGGTGGT	CCGCTGCTGTAGTTGTATT	TGGAAGTGACGATTGCTAACGA

Thermal cycling conditions were the following: 2 min at 50°C, followed by 10 min at 95°C, followed by 45 repeats of 15 s at 95°C and 1 min at 60°C. Data collection was done during each annealing phase. During each run, a standard dilution of the plasmid with known quantity was included to permit gene quantification by means of the supplied software, according to the instructions of the manufacturer. In each run, a negative control (distilled water) was included.

In this study, a relative quantification method was used. The number of copies of cDNA per ml (a measure of the amount of mRNA) was divided by the number of copies of gDNA per ml. This quotient represents the amount of gene expression per viable bacterium.

3.2.7. Statistical analysis

All statistical analyses of the *in vitro* and *in vivo* gene expression data were performed with GraphPad prism (GraphPad software version 4.2, San Diego, USA) as described (Massonet *et al.* 2006;Pintens *et al.* 2008;Vandecasteele *et al.* 2003;Vandecasteele *et al.* 2004). Since the *in vitro* and *in vivo* cDNA/gDNA ratios were not normally distributed at any time point, all data were $\log_{10}$ transformed in order to fulfill the requirements of normality.

For the *in vitro* gene expression data, two hypotheses were tested. A significant change in gene expression levels over time within one group (sessile or planktonic) was tested with a one-way analysis of variance (One-way ANOVA). A significant difference in the evolution over time of the gene expression levels between the sessile group and the planktonic group was tested with a two-way ANOVA. When the one-way ANOVA was significant, two-sided univariate tests with a correction for multiple comparisons were done (Bonferroni test) to locate the significant differences.

For the *in vivo* gene expression data, a one-way ANOVA was used to test if there was a significant evolution of the expression levels over time. When the one-way ANOVA test was significant, the two-sided Bonferroni multiple-comparison method was used to determine which time points differed at $\alpha = 0.05$, with a correction for multiple comparisons.

3.3. Results

3.3.1. Expression of *ses* genes in planktonic and sessile bacteria *in vitro* and *in vivo*

Expression of *ses* genes was compared between sessile bacteria and their planktonic counterparts during *in vitro* biofilm formation at different time points (Table 3.2. and Figure 3.1.). Expression of *ses* genes in sessile bacteria at different time points during *in vivo* FBI in the rat model was compared to the baseline expression level at time point zero (Table 3.3. and Figure 3.2.). Different *ses* genes showed different patterns of gene expression, as are described below:

sesN

The *in vitro* expression of *sesN* significantly decreased in planktonic bacteria after inoculation but stayed relatively constant in sessile bacteria. On the other hand, expression of *sesN* in sessile bacteria was upregulated compared to their planktonic counterparts.

The i*n vivo* expression of *sesN* increased and peaked at 4 h post-implantation. Expression of *sesN* was high during early and late FBI.

sdrG

The *in vitro* and *in vivo* expression pattern of *sdrG* was similar to the expression pattern of *sesN*.

sesO

The *in vitro* expression of *sesO* increased in sessile bacteria 10 min after inoculation and then stayed constant. In planktonic bacteria *sesO* expression first decreased 10 min after inoculation and then increased to the same level of expression as found in sessile bacteria.

The *in vivo* expression of *sesO* was almost constant except for the late FBI during which it was upregulated.

sesE

The *in vitro* expression of *sesE* sharply increased in sessile bacteria immediately after inoculation and stayed constant during the rest of the experiment. The expression of *sesE* increased in planktonic bacteria after 35 min.

The *in vivo* expression of *sesE* was almost constant except during the late FBI during which it was upregulated.

sesL

The *in vitro* expression of *sesL* in planktonic bacteria stayed constant throughout the experiment, but the *sesL* expression in sessile bacteria gradually increased and peaked 1 h after inoculation and stayed constant during the rest of the experiment.

The *in vivo* expression of *sesL* was upregulated during the early and late FBI.

sesJ

The *in vitro* expression of *sesJ* in planktonic bacteria decreased 2 h post-inoculation, whereas inoculation induced a sharp increase in *sesJ* expression in sessile bacteria which peaked at 35 min, thereafter *sesJ* expression decreased towards the expression level in their planktonic counterparts.

The *in vivo* expression of *sesJ* was upregulated during the early and late FBI.

sesK

The *in vitro* expression of *sesK* in planktonic bacteria was constant throughout the experiment, whereas *sesK* expression in sessile bacteria increased 1 h post-inoculation and stayed constant for the rest of the experiment.

The *in vivo* expression of *sesK* was upregulated during the early and late FBI similar to *in vivo* expression of *sesJ*.

sesP

The *in vitro* expression of *sesP* in planktonic bacteria decreased 10 min post-inoculation, whereas in sessile bacteria it first increased between 10-35 min and then decreased towards the *sesP* expression level found in planktonic bacteria.

The *in vivo* expression of *sesP* was downregulated during the early FBI and was constant during the rest of the experiment time.

sesM

The *in vitro* expression of *sesM* in both planktonic and sessile bacteria decreased after inoculation and stayed constant.

The *in vivo* expression of *sesM* was upregulated during the early FBI and was constant during the rest of the experiment time.

sesB

The *in vitro* expression of *sesB* in planktonic bacteria was constant, whereas inoculation induced a sharp increase in *sesB* expression in sessile bacteria which stayed constant during the rest of the experiment.

The *in vivo* expression of *sesB* was downregulated during the early FBI and was constant during the rest of the experiment time.

sesQ

The *in vitro* expression of *sesQ* in planktonic bacteria decreased 1 h post-inoculation, whereas *sesQ* expression in sessile bacteria sharply increased immediately after inoculation and stayed constant during the rest of the experiment.

The *in vivo* expression of *sesQ* was downregulated during the early and late FBI.

sesC

The *in vitro* expression of *sesC* in both planktonic and sessile bacteria first decreased between 10 - 35 min and then increased towards the expression level at time point zero (T = 0).

The *in vivo* expression of *sesC* was upregulated during the early and late FBI but downregulated at day 1.

sdrF

The *in vitro* expression of *sdrF* in planktonic bacteria was constant, whereas in sessile bacteria *sdrF* expression initially increased 10 min post-inoculation and then returned to the expression level of time point zero (T = 0) between 35 min – 2 h and was again increased at time point T=3 h.

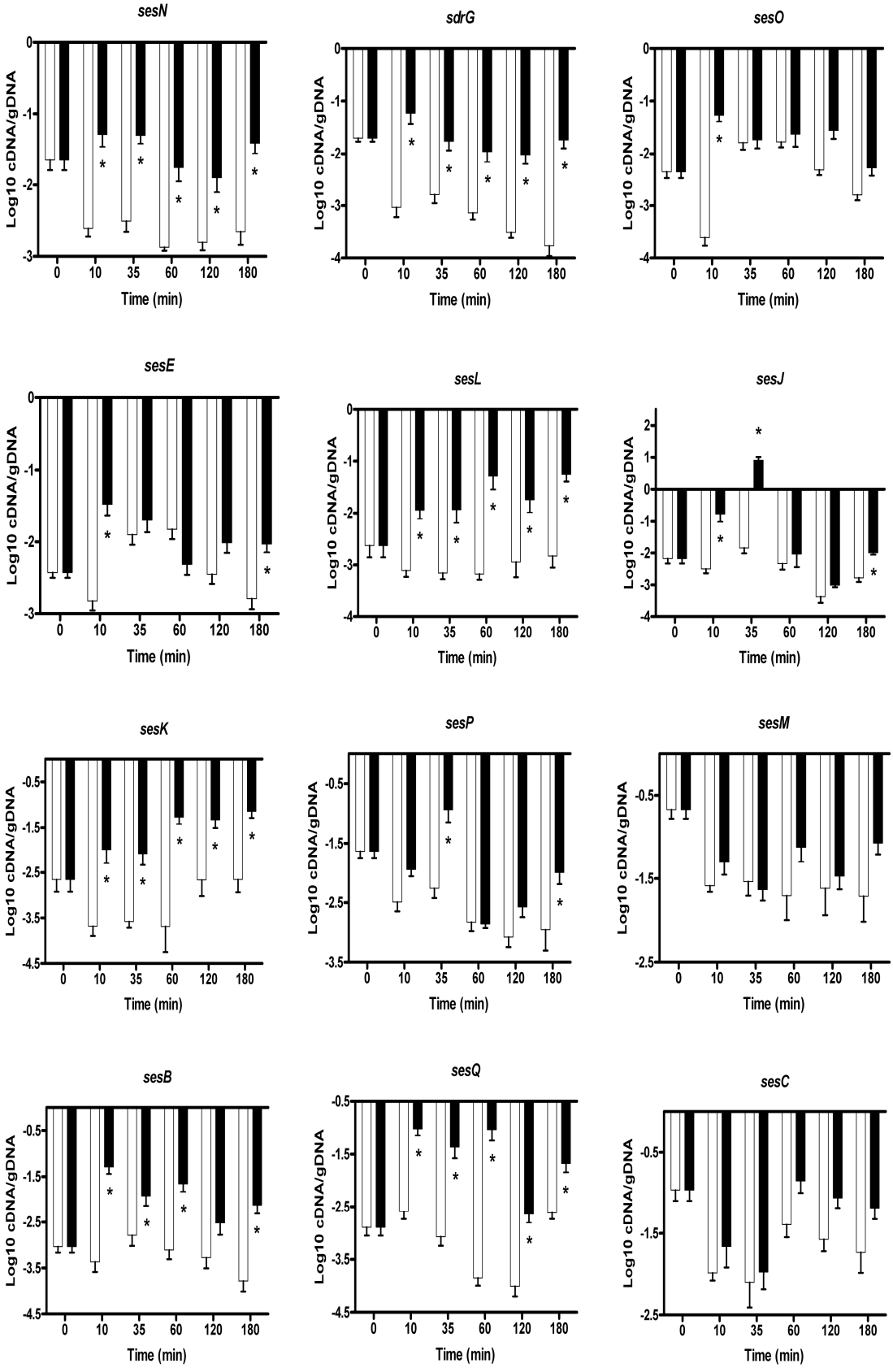
The *in vivo* expression of *sdrF* was unchanged during the early and late FBI except that it increased at the end of experiment (T = 2 weeks).

3.4. Discussion

In this study, the evolution over time of the expression of *S. epidermidis ses* genes during *in vitro* and *in vivo* FBI was investigated. Timewise analysis of gene expression

Table3.2. *In vitro* gene expression pattern of *ses* genes in sessile bacteria compared to their planktonic counterparts. **↑:** Upregulation, **↔:** No changes, **↓:** Downregulation

ses gene	0 min	10 min	35 min	60 min	120 min	180 min
sesN	↔	↑	↑	↑	↑	↑
sdrG	↔	↑	↑	↑	↑	↑
sesO	↔	↑	↔	↔	↔	↔
sesE	↔	↑	↔	↔	↔	↑
sesL	↔	↑	↑	↑	↑	↑
sesJ	↔	↑	↑	↔	↔	↑
sesK	↔	↑	↑	↑	↑	↑
sesP	↔	↔	↑	↔	↔	↑
sesM	↔	↔	↔	↔	↔	↔
sesB	↔	↑	↑	↑	↔	↑
sesQ	↔	↑	↑	↑	↑	↑
sesC	↔	↔	↔	↔	↔	↔
sdrF	↔	↔	↔	↔	↔	↑


sesN
sdrG
sesO
sesE
sesL
sesJ
sesK
sesP
sesM
sesB
sesQ
sesC
Log10 cDNA/gDNA
Time (min)
0
10
35
60
120
180

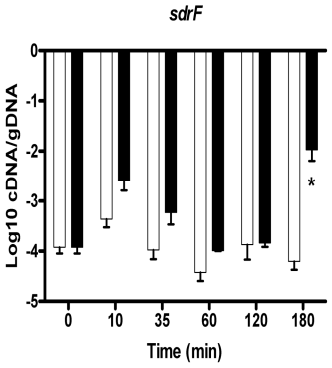
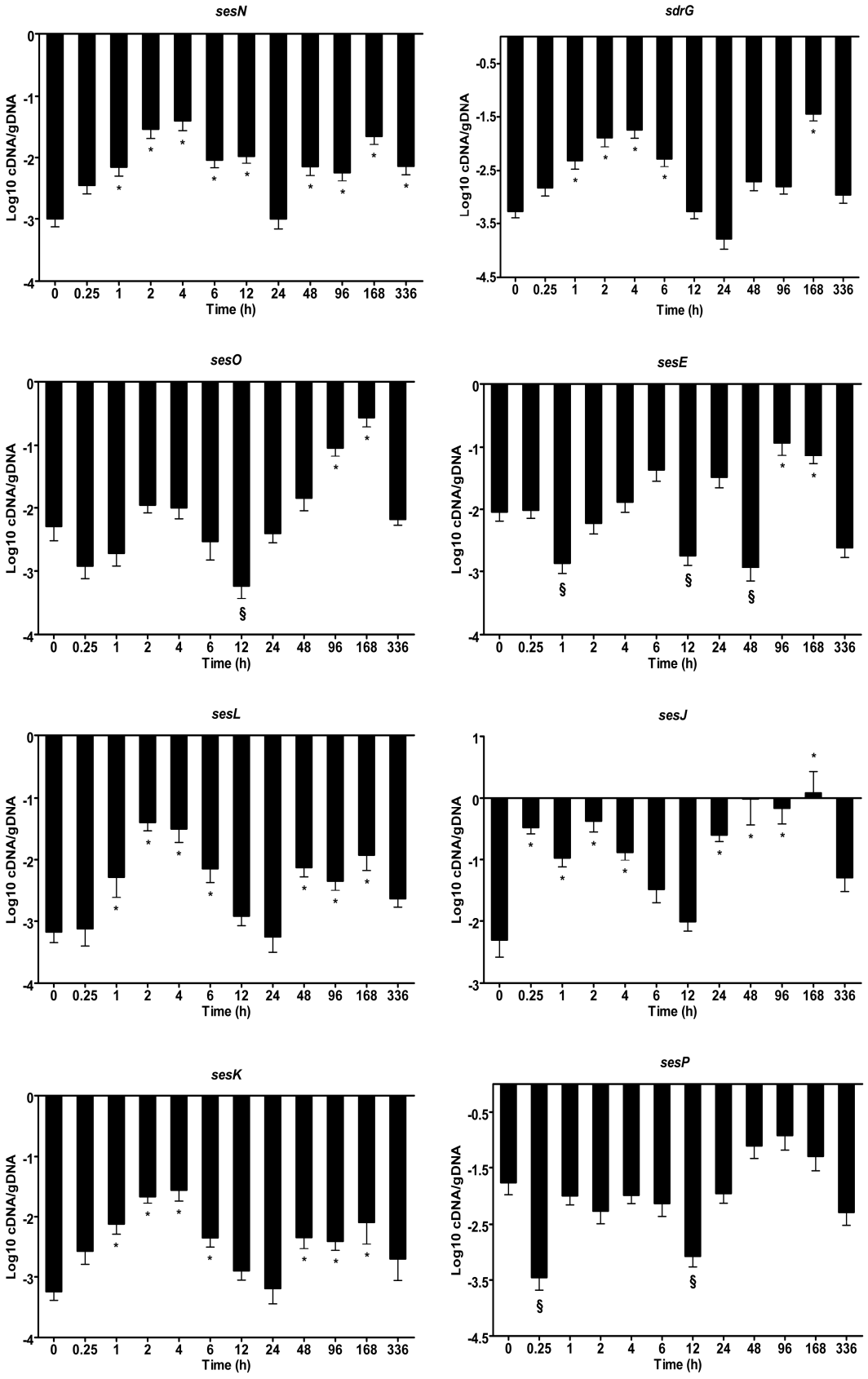


Figure.3.1. *In vitro* expression of *ses* genes in planktonic (□) and sessile (■) bacteria. Gene expression was quantified as the $\log_{10}$ cDNA/gDNA ratio on the Y axis. The error bars represent standard deviations. At each time point 8 samples from two independent experiments were assessed. The X axis indicates time (min). Whenever the gene expression in sessile bacteria compared to their planktonic counterparts was significantly different ($p<0.05$; 2-way-ANOVA) it was marked with a (*) for upregulation and a (§) for downregulation.

Table3.3. *In vivo* gene expression pattern of *ses* genes in sessile bacteria over time compared to time point zero (T = 0). **↑:** Upregulation, **↔:** No changes, **↓:** Downregulation

***ses* gene**	**0 h**	**0.25 h**	**1 h**	**2 h**	**4 h**	**6 h**	**12 h**	**24 h**	**48 h**	**96 h**	**168 h**	**336 h**
sesN	↔	↔	↑	↑	↑	↑	↑	↔	↑	↑	↑	↑
sdrG	↔	↔	↑	↑	↑	↑	↔	↔	↔	↔	↑	↔
sesO	↔	↔	↔	↔	↔	↔	↓	↔	↔	↑	↑	↔
sesE	↔	↔	↓	↔	↔	↔	↓	↔	↓	↑	↑	↔
sesL	↔	↔	↑	↑	↑	↑	↔	↔	↑	↑	↑	↔
sesJ	↔	↑	↑	↑	↑	↔	↔	↑	↑	↑	↑	↔
sesK	↔	↔	↑	↑	↑	↑	↔	↔	↑	↑	↑	↔
sesP	↔	↓	↔	↔	↔	↔	↓	↔	↔	↔	↔	↔
sesM	↔	↔	↑	↑	↑	↔	↔	↔	↔	↔	↔	↔
sesB	↔	↔	↓	↓	↔	↔	↓	↔	↔	↔	↔	↔
sesQ	↔	↓	↓	↔	↔	↔	↓	↔	↔	↓	↔	↔
sesC	↔	↔	↑	↑	↑	↔	↔	↓	↔	↔	↑	↔
sdrF	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↑


sesN
sdrG
sesO
sesE
sesL
sesJ
sesK
sesP
Log10 cDNA/gDNA
Time (h)
0 0.25 1 2 4 6 12 24 48 96 168 336
*
§

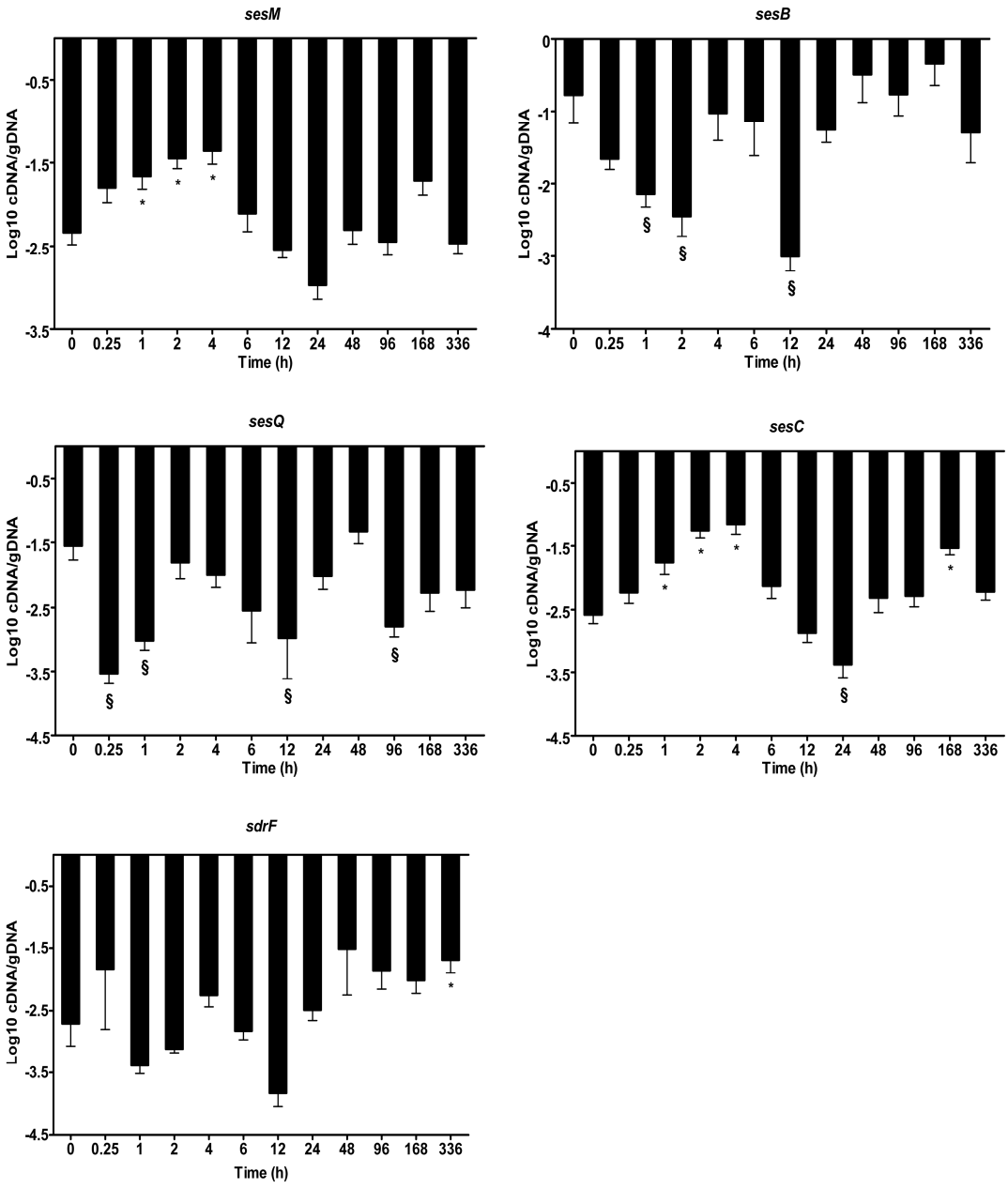


Figure.3.2. *In vivo* levels of expression of *ses* genes over time for two weeks after implantation. The expression level at any time point is represented as the $\log_{10}$ cDNA/gDNA ratio (Y axis) versus time (h) on the X axis. The error bars represent standard deviations. At each time point 16 samples from 2 independent experiments were assessed. Significant differences ($p<0.05$; 1-way-ANOVA) between any given time point, and time point zero (T=0) were marked by (*) for upregulation and (§) for downregulation.

during the course of biofilm development delivers significant information on the role of the genes studied (Massonet *et al.* 2006;Pintens *et al.* 2008;Vandecasteele *et al.* 2003;Vandecasteele *et al.* 2004). However, gene expression studies cannot replace the information acquired with mutagenesis.

The methodology used for gene expression studies has been shown to be sufficiently sensitive to document the time-dependent induction *in vitro* and *in vivo* of biofilm genes. Using this methodology, it has been shown that; i) the expression of *ica* operon genes was high during the colonization and establishment of biofilm formation (Vandecasteele *et al.* 2003), ii) *aap* expression was only upregulated during the accumulation phase (Pintens *et al.* 2008;Vandecasteele *et al.* 2003), iii) the expression levels of alkaline shock protein 23 (*asp23*) which is solely dependent on SigB, mirrored the expression pattern of *sigB* in both planktonic and sessile bacteria (Pintens *et al.* 2008), iv) the amount of 16S rRNA per genome as a determinant of metabolic activity was very low during late *in vivo* FBI which could explain at least in part resistance of biofilms to antimicrobial agents (Vandecasteele *et al.* 2004), v) the expression of *sirR* and the regulatory effect of *sirR* on the *sitABC* operon (iron-responsive genes) were different in planktonic and sessile bacteria (Massonet *et al.* 2006).

Some general principles can be deduced from the gene expression patterns of genes with a proven role in biofilm formation such as *ica* operon genes, *aap*, and 16S rRNA. The sharp increase in expression of a gene after contact with the foreign body *in vitro* and *in vivo* might indicate a possible role of that gene in foreign body colonization. High levels of expression of a gene during the early and late *in vivo* FBI might be attributed to the importance of that gene in the establishment or maintenance of the biofilm, respectively. However, the increase in expression of a gene after the contact with the foreign body *in vitro* and *in vivo* might be either a direct consequence of the contact with the foreign body or mediated by regulatory systems. Expression of staphylococcal virulence factors and surface proteins is regulated by two previously identified regulatory loci, *agr* and *sarA* (Gill *et al.* 2005). The *agr* system in *S. aureus* and *S. epidermidis* downregulates the synthesis of surface proteins in response to changes in bacterial cell density at the onset of the stationary growth phase (Vuong *et al.* 2000). This might

partially explain why most of these genes were downregulated at time points 12 h and 24 h.

The sharp increase in *sesO*, *E*, *J*, *B*, *Q* expression in sessile bacteria *in vitro* after exposure to the foreign body, may lead to the assumption that these genes play a role in primary attachment. However, *in vivo* expression data do not confirm the *in vitro* data for these genes. This difference can be due to the different *in vivo* and *in vitro* environmental factors that can affect the expression of genes or the different factors which are important for attachment of bacteria to abiotic (*in vitro* condition) or biotic surface (*in vivo*) that is rapidly coated with extracellular matrix proteins (ECM) of the host. The *in vitro* increase in *sesL*, *K*, *P* expression in sessile bacteria occurred not immediately after the exposure to the foreign body and was not confirmed by *in vivo* gene expression results.

Due to the lack of information about the role of these genes in biofilm formation, it is difficult to interpret *in vitro* gene expression patterns of *ses* genes. However, our data at least confirm previously reported results that showed upregulation and expression of *sesN*, *sdrG*, *sesE*, *sesB*, *sesC*, and *sdrF* genes in *S. epidermidis* biofilm-associated bacteria (Arrecubieta *et al.* 2007;Sellman *et al.* 2008;Yao *et al.* 2005a).

The *in vivo* gene expression data suggest a role for *sesN*, *G*, *L*, *J*, *K*, *M*, *C* in the establishment of *in vivo* FBI, due to their upregulation in early FBI. Upregulation of *sesN*, *O*, *E*, *L*, *J*, *K*, *C*, *F* during the late *in vivo* FBI suggests a possible role for these genes in maintenance and persistence of *in vivo* FBI. *sesN*, *L*, *J*, *K*, *C* are genes which might play a role in the entire course of *in vivo* FBI, whereas the expression of *sesP*, *B*, *Q* was not upregulated during early or late *in vivo* FBI.

3.5. Conclusion

Our data show that the selected genes are expressed in biofilm cells during *in vitro* and *in vivo* biofilm formation and confirm some previously reported gene expression data. However, additional functional study is necessary to explain the different *in vitro* and *in vivo* gene expression patterns.

CHAPTER 4:

EFFECT OF RABBIT POLYCLONAL ANTI-SES ANTIBODIES ON *S. EPIDERMIDIS* BIOFILM FORMATION *IN VITRO*

4. Effect of rabbit polyclonal anti-Ses antibodies on *S. epidermidis* biofilm formation *in vitro*

4.1. Introduction

The *in vitro* and *in vivo* expression patterns of *ses* genes varied widely. Therefore and also due to the unknown role of the selected genes in biofilm formation, gene expression data alone were not sufficient for the selection of the best potential targets. Hence, we chose 5 out of 13 Ses proteins for further *in vitro* biofilm inhibition studies. The 5 proteins chosen include 3 LPXTG proteins with an uninvestigated role in biofilm formation and the 2 largest ABC transporters also with unknown function. We cloned, expressed and purified the extracellular part of these 5 selected Ses proteins. The recombinant Ses proteins (rSes) and ethanol killed bacteria [whole cell (WC)] were used to immunize rabbits. Total IgG from pre- and hyperimmune sera samples were purified and used in a semi-quantitative microtiter plate method (Christensen *et al.* 1985;Sun *et al.* 2005) to evaluate the effect of IgG antibodies on *S. epidermidis* biofilm formation.

4.2. Materials and methods

4.2.1. Construction and purification of histidine-tagged fusion proteins

Recombinant extracellular domains of the Ses proteins were expressed with hexahistidine tags at their N or C termini using the expression vector pET11c (Stratagene, LaJolla, CA). Each *ses* gene fragment was amplified using forward and reverse primers for each *ses* gene (Table 4.1.) which incorporate flanking *Nhe*I and *Bam*HI restriction sites (*in italics and underlined*) and a sequence coding for a N or C-terminal His^6 tag (**in bold**). Amplicons were cloned in pET11c and the resulting plasmids [pET11c*sesL*, *K*, *M*, *B*, *C*] were transformed into *E. coli* BL21 (DE3). The resulting plasmids were used for recombinant protein production.

Recombinant truncated Ses proteins were expressed in *E. coli* BL21 (DE3) using the expression vector pET11c containing fragments of *ses* genes as previously described (Hermans *et al.* 2006). Briefly, after transformation, *E. coli* BL21 (DE3) was grown with shaking (250 rpm) at 37ºC in Luria-Bertani (LB) broth with 100 µg/ml ampicillin to an OD_{600}nm (optical density at 600 nanometer) of 0.6-1.0. Expression was induced by addition of 1 mM IPTG for 2 h. After cooling on ice, cells were harvested by centrifugation (4000 rpm, 10 min at 4ºC) and resuspended in 5 ml imidazole buffer (20 mM phosphate, 0.5 M NaCl, 10 mM imidazole) and frozen at -20ºC. Cells were first thawn on ice and then sonicated 3 times for 30 s. After centrifugation (30 min, 15000 rpm at 4ºC) the supernatant was used for Ni^+ affinity chromatography purification of the recombinant proteins with the HisTrap™ Kit (Amersham Pharmacia, Uppsala, Sweden). The columns were washed with 40 mM imidazole buffer and proteins were eluted with 300 mM imidazole buffer. The purified recombinant protein was dialyzed against 10 mM HEPES buffer, pH 7.5, freeze dried, and stored at -20 °C. From one liter of *E. coli* culture 70 mg recombinant protein was purified. Purity of the recombinant proteins was determined by Coomassie blue staining of SDS-PAGE gel electrophoresis. The sequence of the purified peptide was confirmed by amino acid analysis and MALDI-TOF mass spectrometry.

4.2.2. Preparation and purification of polyclonal anti-rSes proteins IgG antibodies

Polyclonal antibodies were produced at Eurogentec (Seraing, Belgium) by immunization of rabbits with purified rSes proteins according to standard immunization protocols. Ethanol (80%) killed *S. epidermidis* ATCC 12228 were also used as whole cell preparation to raise serum against the complete surface of *S. epidermidis.* Briefly, specific pathogen free rabbits were immunized with 100 µg rSes proteins or killed bacteria in Complete Freund's Adjuvant (CFA) and boosters were given at 14, 28 and 56 days after the first immunization with 50 µg antigens in Incomplete Freund's Adjuvant (IFA) intraperitoneally. Pre-immune serum was taken before the first immunization. After 87 days, the total blood of the rabbit was collected and the serum was stored at –20°C.

Total IgGs from pre- and post-immune sera were purified by absorption to a protein-G column (GE-Healthcare) according to the manufacturer's instructions. Purity

Table 4.1. List of primers used for cloning in pET11c for production of recombinant Ses proteins.

***ses* gene**	**Forward primer 5'… 3'**	**Reverse primer 5'… 3'**
sesL	CACGT*GCTAGC***CATCACCATCACCATCAC**A AAACGCAAGATGAAGCGAAA	GGAACTCAAATTATTTATTAA*GGATCC*GCAT
sesK	CACGT*GCTAGC*GCTGAATCAAACACTTCAGT TTCTTCT	CTATTACCAAATACAGGTATG***CATCACCATCACC ATCAC***TA*GGATCC*GCAT
sesM	ACTG*GCTAGC***CATCACCATCACCATCAC**GG GGGCACCTCAAGTACAG	GTTACACCAGAATCTATCTATTA*GGATCC*GCAT
sesB	ACGT*GCTAGC*GCAGCCGAAGTAACATCTC	CTCAATTCATACTACGTAGGT**CATCACCATCACC ATCAC**TA*GGATCC*GCAT
sesC	ACGT*GCTAGC*GCAGATTCAGAAAGTACATC	GAACAGCTACAGCTGAT**CATCACCATCACCATC AC**TA*GGATCC*GCAT

and reactivity of the purified IgGs against their respective rSes proteins was determined by Coomassie blue staining of SDS-PAGE gel electrophoresis and with ELISA and Western blot according to standard protocols.

4.2.3. Recognition of Ses proteins by rabbit anti-Ses sera

To evaluate the presence of the respective proteins on the surface of *S. epidermidis,* an indirect ELISA and Western blot with the pre- and post-immune sera on purified recombinant protein and lysed *S. epidermidis* ATCC 12228 were performed according to standard protocols. The ELISA was considered positive when the OD of post-immune sera was at least twice as high as the OD of pre-immune sera.

4.2.4. *In vitro* biofilm inhibition assays

The effect of pre- and post-immune IgGs against rSes proteins on *in vitro* biofilm formation during the first 2 h, considered as primary attachment and overnight (14 h) was studied. For quantification of biofilms, 20 µl of frozen cultures of *S. epidermidis* strain 10b were inoculated into 5 ml BHI and grown to the end-exponential growth phase in a

shaking incubator at 37°C. Cultures were subsequently diluted to an OD_{600} of 0.005 (5×10^6 CFU/ml) in fresh BHI medium.

To evaluate the effect of IgGs on primary attachment of *S. epidermidis* strain 10b, starting cultures were diluted to an OD_{600} of 0.005 and subsequently grown at 37°C to an OD_{600} of 1. Cultures then were mixed with either pre- or post immune IgGs (10 µg/ml) and after 2 h incubation at 4°C, 200 µl of the mixtures were pipetted into 96-well polystyrene microtiter plates (BD Biosciences, Heidelberg, Germany) and incubated for 2 h at 37°C without shaking.

To study the effect of IgGs on biofilm formation during 14 h, the cultures diluted to an OD_{600} of 0.005 were mixed with either pre- or post-immune IgGs (10 µg/ml) and after 2 h incubation at 4°C, 200 µl of the mixtures (10^6 cells per well) was added to each well of 96-well polystyrene microtiter plates**,** and incubated overnight at 37°C without shaking.

After the incubation, plates were washed 3 times with phosphate buffered saline (PBS) (pH 6.8, containing 0.5 M NaCl and 10 mM EDTA) and adherent biofilms were stained with 200 µl of 1% (wt/vol) crystal violet (Sigma) for 10 min, after which the plates were washed 3 times with water and dried. For quantification, 160 µl of 30% (vol/vol) acetic acid was added to each well to dissolve the stain. The OD_{595} of the dissolved stain was measured in a multipurpose UV/VIS plate reader**.** Percent inhibition of biofilm formation was calculated by using the following formula: $(A_{595,\ positive} - A_{595,\ antibody})/(A_{595,\ positive} - A_{595,\ negative}) \times 100\%$ (Sun *et al.* 2005). Average inhibition at each concentration of IgGs was obtained from at least 8 independent measurements generated in at least 2 independent experiments. *S. epidermidis* strain 10b in BHI without any added IgGs was used as positive control, and BHI without bacteria was used as negative control.

4.2.5. Statistical analysis

For all data from bacterial adherence assays, two hypotheses were tested. A significant change in adherence levels by different IgGs within one group (pre-immune or anti-Ses IgGs) was tested with a one-way analysis of variance (1-way ANOVA). A significant difference in adherence with different IgGs between the pre-immune IgGs and anti-Ses IgGs groups was tested with a two-way ANOVA. When the one-way ANOVA

was significant, two-sided univariate tests with a correction for multiple comparisons were done (Bonferroni test) to locate the significant differences.

4.3. Results

4.3.1. Recognition of Ses proteins on the surface of *S. epidermidis*

Western blot analysis was positive on both recombinant protein and bacterial lysate for all anti-Ses sera except SesK where only the recombinant protein was recognized. Similarly, all recombinant proteins except for SesK were recognized by whole cell antisera. ELISA on their respective recombinant proteins and lyzed *S. epidermidis* ATCC 12228 was positive for all anti-Ses sera, whereas ELISA on whole cells was positive for anti-SesL, B, C sera and negative for anti-SesK, M sera. ELISA was negative for all pre-immune sera (Table 4.2.).

Table 4.2. Western blot and ELISA results of immune sera against recombinant Ses proteins and whole cell *S. epidermidis* ATCC 12228. **WC:** whole cell

	on respective rSes protein		on *S. epidermidis* lysate		on WC *S. epidermidis*
Antiserum against	**Western blot**	**ELISA**	**Western blot**	**ELISA**	**ELISA**
SesL	+	+	+	+	+
SesK	+	+	-	+	-
SesM	+	+	+	+	-
SesB	+	+	+	+	+
SesC	+	+	+	+	+
WC	+***	+	+	+	+

***: Western blot with whole cell antiserum on recombinant proteins was positive for all proteins except SesK

4.3.2. Biofilm inhibition by purified polyclonal anti-rSes IgGs

The maximum inhibition of initial attachment of the bacteria to the polystyrene surface compared to the controls (bacteria pre-incubated with pre-immune IgGs or without IgGs) was caused by the total IgGs purified from serum of the rabbit immunized with rSesC [95% confidence interval (CI) of diff. 7.646 to 44.49; *p<0.01*; as determined by a Bonferroni test] (Figure 4.1.).

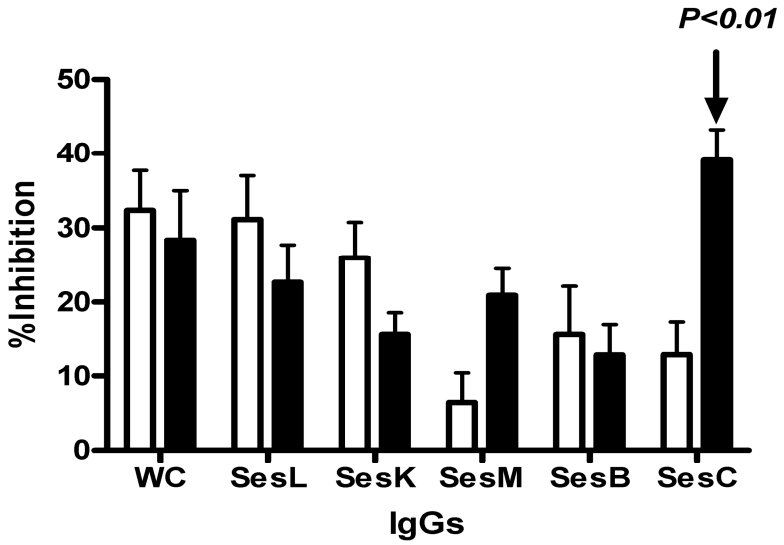


Figure 4.1. Effect of total IgGs purified from pre-immune (□) and anti-Ses (L, K, M, B, C) and anti-WC sera (■) on primary attachment of *S. epidermidis* 10b to polystyrene surface. Bacteria were mixed with IgGs and incubated for 2 h at 4°C and then pipetted into the wells. After 2 h incubation at 37°C, plates were washed and stained with crystal violet and the OD_{595} was measured. The error bars indicate standard deviations. Data are the average of 8 measurements in 2 independent experiments. Biofilm inhibition was defined in the text. **WC:** whole cell

A 2 h pre-incubation of bacteria with different IgGs followed by an overnight (14 h) incubation in the 96-well polystyrene microtiter plates showed that the maximum inhibition of biofilm formation was caused again by total IgGs purified from rSesC induced rabbit antisera (Figure 4.2.). However, this reduction was not statistically significant.

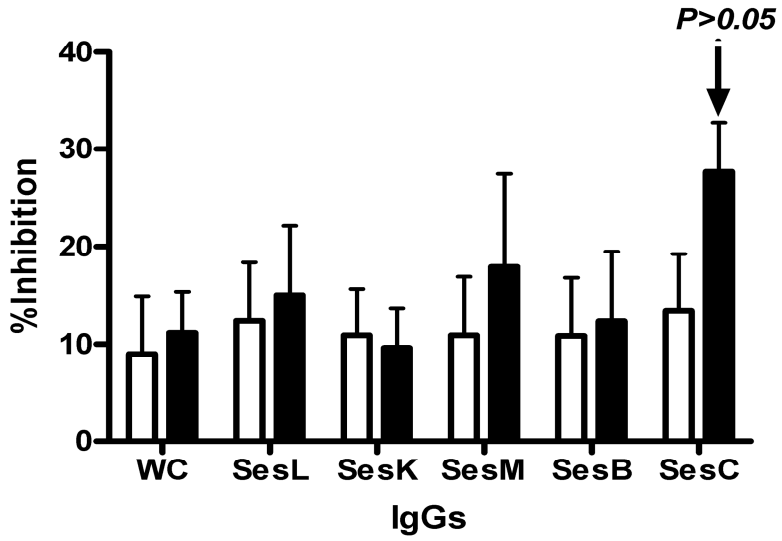


Figure 4.2. Inhibition of *S. epidermidis* 10b biofilm formation by total IgGs purified from pre-immune (□) and anti-Ses (L, K, M, B, C) and anti-WC sera (■). Overnight culture were diluted to an OD_{600} of 0.005, mixed with the IgGs and incubated for 2 h at 4°C and then pipetted into the wells and incubated overnight (14 h) at 37°C. Formation of biofilm was measured with crystal violet stain and biofilm inhibition was calculated as was explained in the text. The error bars indicate standard deviations. Data are the average of at least 8 independent measurements in 2 independent experiments. **WC:** whole cell

4.4. Discussion

Using ELISA and Western blotting techniques, it has been shown that the selected proteins are surface-exposed except for SesK and SesM which might be surface-exposed proteins that are expressed under specific, unknown circumstances.

The *in vitro* expression of *sesL*, *K*, *B* genes was upregulated in sessile bacteria compared to their planktonic counterparts. However, the sharp increase after exposure to the foreign body was only induced in *sesB*. The *in vitro* expression of *sesM*, *C* decreased after the exposure to foreign body and was similar in sessile and planktonic bacteria. During the first 4 h after the implantation of catheter fragments in rats the *in vivo* expression of *sesL*, *K*, *M*, *C* genes increased whereas *sesB* expression decreased (Chapter 3.3.).

Based on the gene expression data we expected that anti-SesB IgGs would show the highest effect on primary attachment. However, as it has been previously explained (Chapter 3.4.), interpretation of gene expression patterns without knowing the function of the protein encoded by that gene is not easy. Whether the proteins predicted to be surface-exposed are true surface-exposed proteins or if after translation and translocation the level of proteins on the surface is correlated with the gene expression levels are important questions that make the interpretation of gene expression results difficult.

Most likely, whole killed bacteria are not suitable antigens for antibody-mediated inhibition of biofilms. Although antibodies to the whole surface fraction of planktonic bacteria may opsonize the bacteria for phagocytosis, it is possible that such polyclonal antibodies inhibit the function of surface proteins such as AgrC which in turn repress the agr activity which in turn increases expression of colonizing factors and induce biofilm formation.

There are several explanations for the low effect of anti-Ses IgGs on *S. epidermidis* biofilm formation: i) expression of proteins on the surface is low, ii) polyclonal antibodies cross react with other non-specific antigens, iii) the concentration of antibodies which was used was not sufficient for inhibition of the function of some Ses proteins. However, since our aim was to identify the best potential target for further investigation, we didn't test different concentrations of different anti-Ses IgGs.

4.5. Conclusion

Based on the effect of different IgGs on primary attachment and inhibition of biofilm formation, SesC was selected for further investigation.

CHAPTER 5:

EXPRESSION OF SESC PROTEIN ON THE SURFACE OF PLANKTONIC AND SESSILE BACTERIA

5. Expression of SesC protein on the surface of planktonic and sessile bacteria

This chapter is based on the following reference: Mohammad Shahrooei, Vishal Hira, Rita Merckx, Benoit Stijlmans, Peter W.M. Hermans, Johan Van Eldere. 2009. Inhibition of *Staphylococcus epidermidis* biofilm formation by rabbit polyclonal antibodies against SesC protein. Infection and Immunity. 77(9):3670-8.

5.1. Introduction

SesC was selected as the best target antigen based on the effect of total IgGs purified from serum of rabbit immunized with rSesC on *S. epidermidis* biofilm formation *in vitro*. In order to validate the *in vitro sesC* expression pattern (Chapter 3), conventional fluorescence microscopy was used to confirm SesC protein expression on the surface of sessile and planktonic *S. epidermidis* bacteria *in vitro*.

5.2. Materials and methods

5.2.1. Purification of polyclonal specific anti-SesC IgG antibodies

In order to enrich for polyclonal specific anti-SesC IgGs (αSesC-IgGs), the rSecC antigen was covalently coupled to the MiniLeak (Medium) affinity resin (Kem-En-Tec, Copenhagen, Denmark) as recommended by the manufacturer. In total, 1 mg rSecC was coupled to 1 ml resin and the coupling efficiency was measured as described by the manufacturers. Subsequently, 5 ml of purified IgG was incubated for 3 h at room temperature with 1 ml of immunoaffinity resin and was subsequently packed into a column. After washing the resin with 100 ml of PBS, immunoabsorbed material was eluted with 0.1 M glycine-HCl buffer, pH 2.7 and immediately dialysed against PBS. After dialysis, the concentration was determined spectrophotometrically at OD_{280} (1.4 OD=1 mg/ml). Purity of the IgGs was determined by Coomassie blue staining of SDS-PAGE gel electrophoresis.

The affinity of IgGs purified from pre-immune serum, and polyclonal αSesC-IgGs isolated from antiserum against rSesC was quantified with an alkaline phosphatase conjugated anti-rabbit immunoglobulin by indirect protein ELISA according to standard protocols.

5.2.2. *In vitro* protein expression studies

In order to validate the *in vitro sesC* expression pattern (Chapter 3), conventional fluorescence microscopy was used to confirm SesC protein expression on the surface of sessile and planktonic *S. epidermidis* bacteria *in vitro*. Preparation of samples for fluorescence microscopy was similar to preparation of samples for gene expression analysis *in vitro* with the exception that samples were taken at different time points (0, 30, 60, 90 and 120 min) after inoculation and sessile cells were separated from catheter fragments by sonication for 10 min at 40 kHz in a water bath (Branson 2200, Branson Ultrasonics). The bacteria were fixed using PBS containing 1.5% formaldehyde and 0.5% glutaraldehyde for 30 min, and washed with PBS. Next, the bacteria were pre-incubated for 20 min on ice with 2.4G2 (Fc-blocking antibody, BD Pharmingen), after which αSesC-IgGs were added at 5 µg per 100 µl for another 30 min. Subsequently, the bacteria were washed twice with ice-cold PBS and FITC-labeled goat-anti-rabbit antibodies (BD-Pharmingen) was added for 30 min. Finally, the cells were washed twice with PBS and viewed with a fluorescence microscope (Leica, Germany) equipped with an oil-immersion plan neofluar objective (100X; numeric aperture=1.25).

The following samples were used as negative controls: 1) cells incubated with pre-immune IgGs and FITC-labeled goat-anti-rabbit antibodies, 2) cells incubated with polyclonal rabbit antibody against mouse immunoglobulins and FITC-labeled goat-anti-rabbit antibodies, 3) cells incubated with only FITC-labeled goat-anti-rabbit antibodies.

5.3. Results

5.3.1. Affinity of αSesC-IgGs to SesC protein

Binding of the purified pre-immune IgGs and of the αSesC-IgGs was tested via an indirect ELISA against the 65 kDa rSesC. Increasing concentrations of purified pre-immune IgGs and αSesC-IgGs showed no binding of pre-immune IgGs and a dose-dependent binding of the purified anti-SesC IgGs (Figure 5.1.).

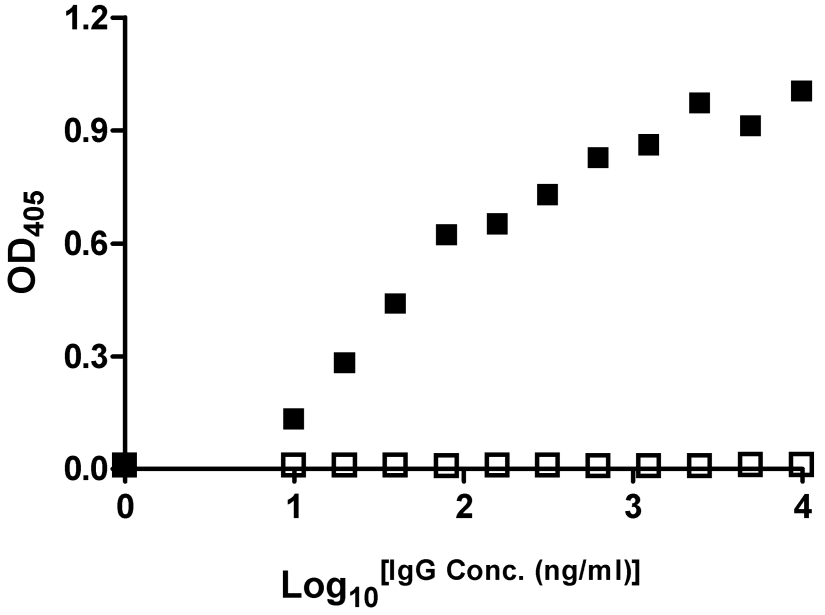


Figure 5.1. Affinity of pre-immune (□) and αSesC-IgGs (■) to rSesC. An indirect ELISA was performed using a 96-well ELISA plate coated with rSesC. IgGs were added to each well and incubated for 3 h at 37°C. Bound IgGs were measured at OD_{405} with an alkaline phosphatase conjugated anti-rabbit immunoglobulin. X and Y axes indicate $\log_{10}$ [IgG concentration (ng/ml)] and OD_{405} absorbance, respectively.

5.3.2. SesC protein expression in sessile and planktonic bacteria *in vitro*

To confirm the results obtained from gene expression analysis, purified polyclonal αSesC-IgGs and FITC-labelled goat-anti-rabbit antibodies were used in an immunofluorescence assay to study *in vitro* protein expression. Comparison of the images taken by fluorescence microscopy from sessile and planktonic bacteria at different time points confirmed the protein expression of SesC on the surface of both sessile and

planktonic *S. epidermidis* 10b bacteria. Visual comparison of images taken at different time points suggested a higher level of fluorescence, in sessile compared to planktonic bacteria (Figure 5.2.). No quantitative measurements were however performed. These data show that gene expression patterns of *sesC* correlate with SesC protein expression.

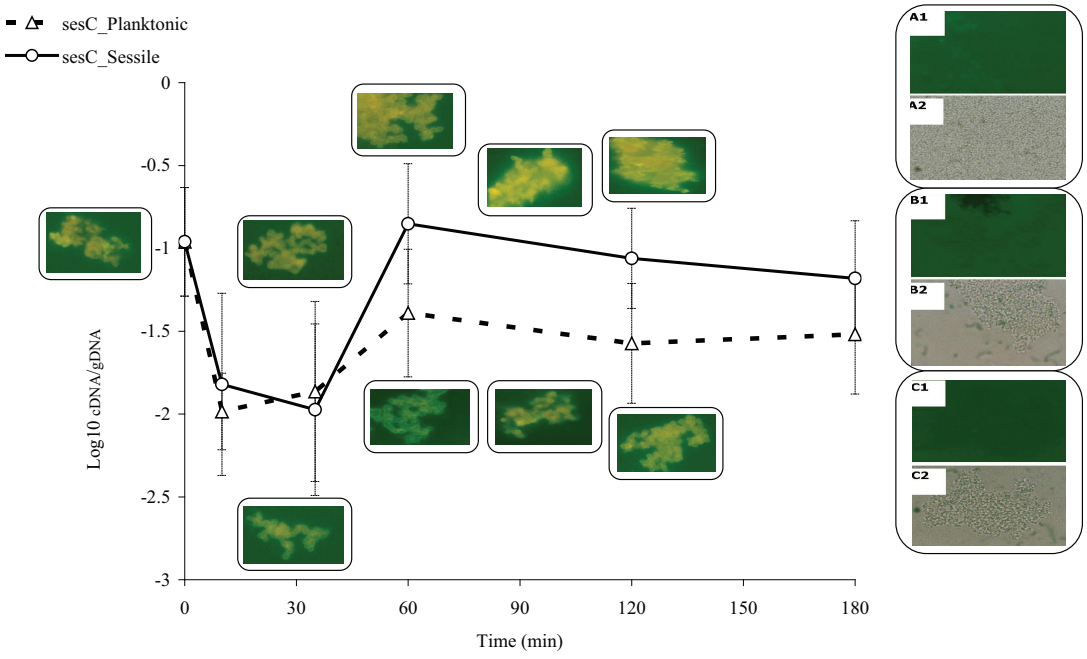


Figure 5.2. *In vitro sesC* gene expression and extracellular presence of the SesC protein in planktonic and sessile bacteria. Gene expression is quantified as the $\log_{10}$ cDNA/gDNA ratio on the Y axis. The error bars represent standard deviations. At each time point 8 samples from 2 independent experiments were assessed. The X axis indicates time (min).

Fluorescence microscopy images are from sessile bacteria (top) and planktonic bacteria (bottom) at different time points. Images A1 and A2 show bacterial cells plus pre-immune IgGs and FITC-labelled goat-anti-rabbit antibodies. Images B1 and B2 are from control samples containing bacterial cells plus polyclonal rabbit antibody against mouse immunoglobulins and FITC-labelled goat-anti-rabbit antibodies. Images C1 and C2 are from control samples of bacterial cells plus only FITC-labelled goat-anti-rabbit antibodies. A1, B1 and C1 were visualized by fluorescence while A2, B2 and C2 were visualized by bright-field microscopy.

5.4. Discussion

Protein expression studies showed that SesC is an extracellular protein that is expressed on the surface of both planktonic and sessile bacteria. Comparison of the images taken by fluorescence microscopy from sessile and planktonic bacteria suggested a higher level of fluorescence staining (meaning protein expression) in sessile bacteria compared to their planktonic counterparts. There was also a higher gene expression level in sessile compared to planktonic bacteria, however this was not statistically significant. Quantification of the difference in protein expression between sessile and planktonic bacteria was not performed because it was not important for our study.

5.5. Conclusion

In conclusion, SesC is a surface-exposed protein of which the expression is slightly higher in sessile cells compared to their planktonic counterparts.

CHAPTER 6:

EFFECT OF SPECIFIC ANTI-SESC ANTIBODIES ON *S. EPIDERMIDIS* BIOFILM FORMATION *IN VITRO*

6. Effect of specific anti-SesC antibodies on *S. epidermidis* biofilm formation *in vitro*

This chapter is based on the following reference: Mohammad Shahrooei, Vishal Hira, Rita Merckx, Benoit Stijlmans, Peter W.M. Hermans, Johan Van Eldere. 2009. Inhibition of *Staphylococcus epidermidis* biofilm formation by rabbit polyclonal antibodies against SesC protein. Infection and Immunity. 77(9):3670-8.

6.1. Introduction

Based on the effect of rabbit polyclonal antibodies on *in vitro S. epidermidis* biofilm formation, the surface-exposed protein SesC was selected as the best potential antigen for further investigation. Prior to an evaluation of whether SesC is a promising target for immunoprophylaxis and immunotherapy against *S. epidermidis* biofilms, the effect of αSesC-IgGs was tested *in vitro* on *S. epidermidis* biofilms at different stages.

Compared to the *in vivo* experiments, the *in vitro* experiments are inexpensive, rapid and easy to perform assays in which the experimental conditions can be easily controlled.

6.2. Materials and methods

6.2.1. Effect of αSesC-IgGs on *in vitro* primary attachment

The effect of pre-immune or αSesC-IgGs on *in vitro* primary attachment was studied using a semi-quantitative adherence assay on 96-well polystyrene microtiter plates as previously described (Chapter 4.2.4.). Briefly, 20 µl of frozen cultures of *S. epidermidis* strains 10b and 1457 were inoculated into 5 ml BHI and grown to the end-exponential growth phase in a shaking incubator at 37°C. Cultures were subsequently diluted to an OD_{600} of 0.005 (5×10^6 CFU/ml) in fresh BHI medium and grown to an OD_{600} of 1. Cultures then were mixed with either pre-immune IgGs or αSesC-IgGs (10 µg/ml) and after 2 h incubation at 4°C, 200 µl of the mixtures were pipetted into 96-well polystyrene microtiter plates and incubated for 2 h at 37°C without shaking. After 2 h

incubation, adherent bacteria were quantified and percent inhibition was calculated as previously described (Chapter 4.2.4.).

6.2.2. Effect of different concentrations of αSesC-IgGs on *in vitro* biofilm formation during 14 h

To study the effect of pre-immune or αSesC-IgGs on biofilm formation during 14 h, the overnight cultures of *S. epidermidis* strains 10b, 1457 and a biofilm-forming *sesC*-negative clinical isolate of *S. warneri* (This study) were diluted to an OD_{600} of 0.005, mixed with either pre-immune IgGs or αSesC-IgGs in concentrations of 1-4 µg/ml. The mixtures were incubated for 2 h at 4°C. 200 µl of the mixtures (10^6 cells per well) was added to each well of 96-well polystyrene microtiter plates**,** and incubated overnight at 37°C without shaking. After 14 h incubation, biofilms were quantified and biofilm inhibition was calculated as described previously (Chapter 4.2.4.).

6.2.3. Effect of αSesC-IgGs on 1-day old biofilms *in vitro*

To evaluate the effect of αSesC-IgGs on 1-day old biofilms of 10b, the diluted overnight cultures of bacteria with an OD_{600} of 0.005 were pipetted into sterile 96-well polystyrene microtiter plates and incubated overnight at 37°C without shaking. After 24 h incubation, growth medium was replaced with fresh medium or fresh medium containing 2.5, 5 or 10 µg/ml pre-immune IgGs or αSesC-IgGs and incubated overnight at 37°C without shaking. After 14 h incubation, the remaining biofilms were quantified and percent detachment was calculated using the same formula used for calculating percent inhibition (Chapter 4.2.4.).

6.2.4. Effect of αSesC-IgGs on biofilm formation of bacteria isolated from 1-day old biofilms

To examine whether the αSesC-IgGs has the same effect on biofilm formation of 10b bacteria isolated from an established biofilm (Sessile origin) as on biofilm formation of planktonic bacteria from an overnight culture, bacteria were isolated from 1-day old biofilms on catheter fragments by sonication for 10 min at 40 kHz in a water bath (Branson 2200, Branson Ultrasonics). The overnight cultures of planktonic bacteria and

suspensions of bacteria isolated from biofilm were adjusted to an OD_{600} of 0.005 and were mixed with αSesC-IgGs (2.5 and 5µg/ml) and after 2 h incubation at 4°C, 200 µl of the mixtures were added to each well (10^6 cells per well) of 96-well polystyrene microtiter plates**,** and incubated overnight (14 h) at 37°C without shaking. Biofilms were quantified and biofilm inhibition was calculated as described previously (Chapter 4.2.4.).

6.2.5. Statistical analysis

Statistical analyses were performed with GraphPad prism 4.2. as previously described (Chapter 4.2.5.) with the exception that a significant change in adherence levels by different concentrations of IgGs within one group (pre-immune or αSesC-IgGs) was tested with a one-way analysis of variance (1-way ANOVA) instead of a significant change in adherence levels by different IgGs within one group (pre-immune or anti-Ses IgGs).

6.3. Results

6.3.1. Effect of αSesC-IgGs on primary attachment

Pre-incubation of strains 10b and 1457 for 2 h with αSesC-IgGs or pre-immune IgGs at 4°C and subsequent incubation for 2 h in the polystyrene wells lead to a significant reduction of initial attachment compared to controls for αSesC-IgGs ($p<0,01$; 1-way-ANOVA), but not for pre-immune IgGs (Figure 6.1.).

6.3.2. Effect of different concentrations of αSesC-IgGs on biofilm formation during 14 h

A 2 h pre-incubation of bacteria with IgGs followed by an overnight (14 h) incubation in the 96-well polystyrene microtiter plates showed that the polyclonal αSesC-IgGs purified from rSesC induced rabbit antisera exhibited a dose-dependent *S. epidermidis* biofilm inhibition activity, whereas the purified IgGs from pre-immune serum showed only low activity that was dose-independent (Figure 6.2.). Increasing the concentration of polyclonal αSesC-IgGs from 1 to 4 µg/ml increased *S. epidermidis*

strains 10b and 1457 biofilm inhibition from 40 to 80% (Figure 6.2.A. & B.). For both strains, the inhibition effects seen with αSesC-IgGs, were significantly different from that obtained with pre-immune IgGs ($p<0.01$; 2-way-ANOVA).

A *sesC*-negative biofilm-positive *S. warneri* strain was included as control; for this strain the inhibitory effect of αSesC-IgGs was not significantly different from that of the pre-immune serum and was not dose-dependent (Figure 6.2.C.). For all 3 strains, the effect of pre-immune IgGs on the biofilm formation was non-significant and dose-independent ($p>0.05$; 1-way-ANOVA).

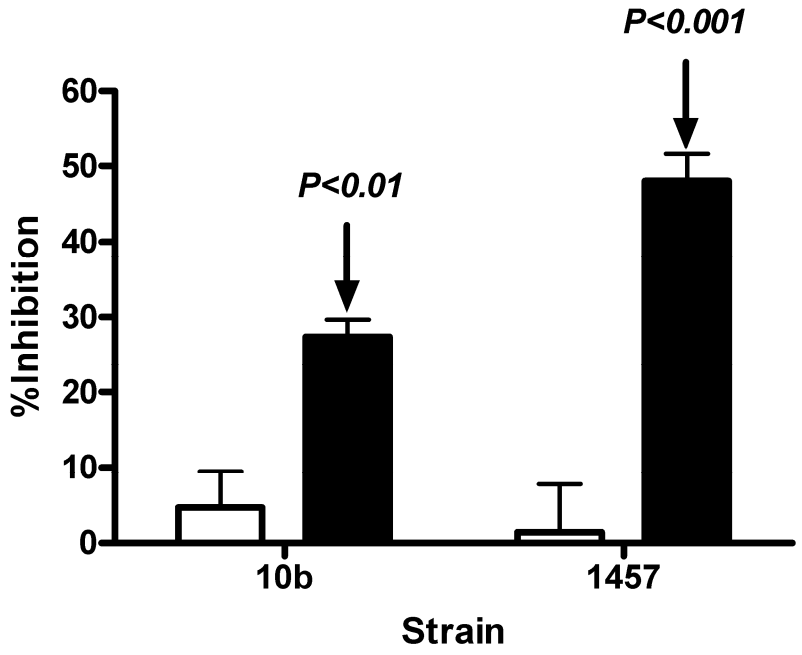


Figure 6.1. Effect of pre-immune (□) and αSesC-IgGs (■) on primary attachment of *S. epidermidis* 10b and 1457 to polystyrene surface. Bacteria were mixed with IgGs and incubated for 2 h at 4°C and then pipetted into the wells. After 2 h incubation at 37°C, plates were washed and stained with crystal violet and the OD_{595} was measured. The error bars indicate standard deviations. Data are the average of 8 measurements in 2 independent experiments.

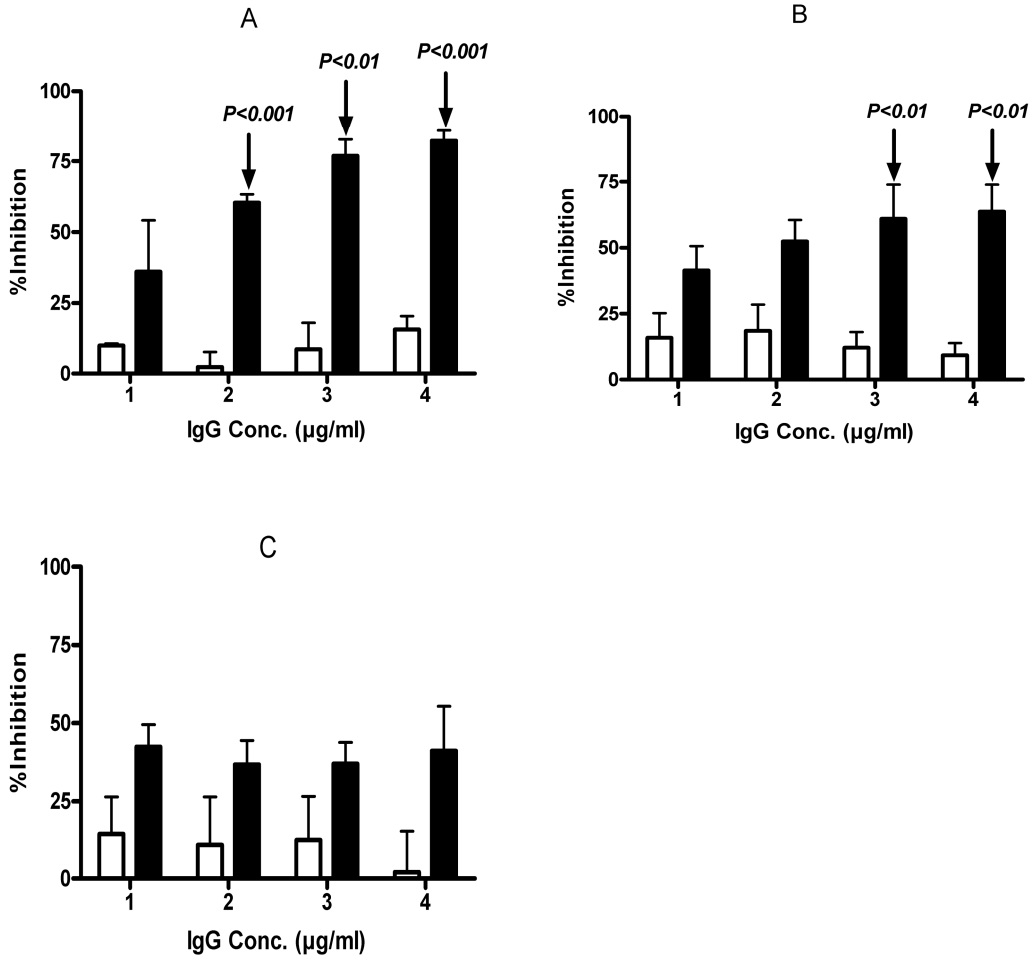


Figure 6.2. Inhibition of biofilm formation by *S. epidermidis* strains 10b (A), 1457 (B) and a *sesC*-negative biofilm-positive clinical isolate of *S. warneri* (C) with increasing concentrations of pre-immune (□) and αSesC-IgGs (■). Overnight cultures were diluted to an OD_{600} of 0.005, mixed with the indicated concentration of IgGs and incubated for 2 h at 4°C and then pipetted into the wells. After 14 h incubation at 37°C, biofilms were quantified at OD_{595}. The error bars indicate standard deviations. At each concentration of IgGs at least 9 independent measurements from 3 independent experiments were assessed. X and Y axes represent concentrations of IgGs (µg/ml) and % biofilm inhibition, respectively.

6.3.3. Effect of αSesC-IgGs on 1-day old biofilms *in vitro*

Addition of αSesC-IgGs on 1-day old biofilms of strain 10b induced a statistically significant dose-dependent decrease in established biofilms compared to biofilms treated with pre-immune IgGs (*p<0,001*; 2-way ANOVA) (Figure 6.3.).

6.3.4. Effect of αSesC-IgGs on biofilm formation by planktonic and isolated bacteria from biofilm

Effect of different concentrations of αSesC-IgGs on biofilm formation of both planktonic and sessile bacteria (isolated from 1-day old biofilms on catheter fragments) was significant, dose-dependent and similar (Figure 6.4.).

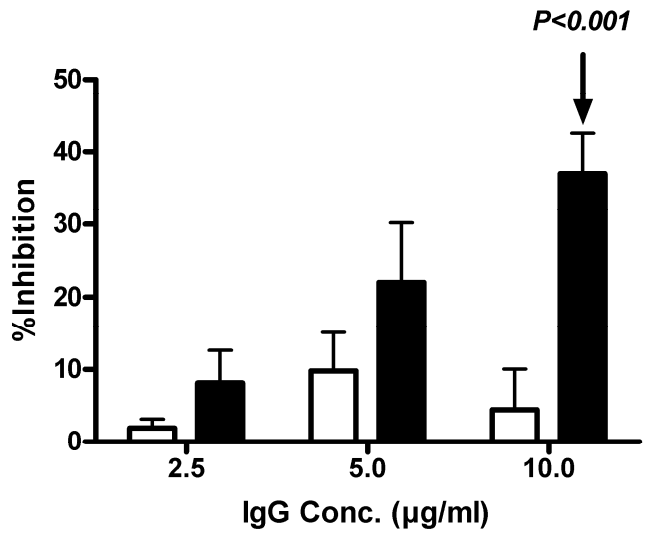


Figure 6.3. Effect of increasing concentrations of pre-immune (□) and αSesC-IgGs (■) on 1-day old biofilms of *S. epidermidis* 10b on polystyrene surface *in vitro*. Growth media after 24 h incubation at 37°C were replaced with fresh BHI (control) or BHI containing 2.5, 5, and 10 µg/ml pre-immune or αSesC-IgGs and then incubated for 14 h at 37°C. Remaining biofilms were stained with crystal violet and quantified at OD_{595} as shown on the Y axis. The X axis represents the IgG concentration (µg/ml). The error bars indicate standard deviations. Data are the average of 8 measurements in 2 independent experiments.

6.4. Discussion

In vitro experiments showed that rabbit polyclonal αSesC-IgGs could significantly reduce primary attachment of *S. epidermidis* to the abiotic surface of polystyrene plates and inhibit biofilm formation by *S. epidermidis* strains 10b and 1457 up to 80% in a dose-dependent manner. In contrast, the effect of rabbit polyclonal αSesC-IgGs on biofilm

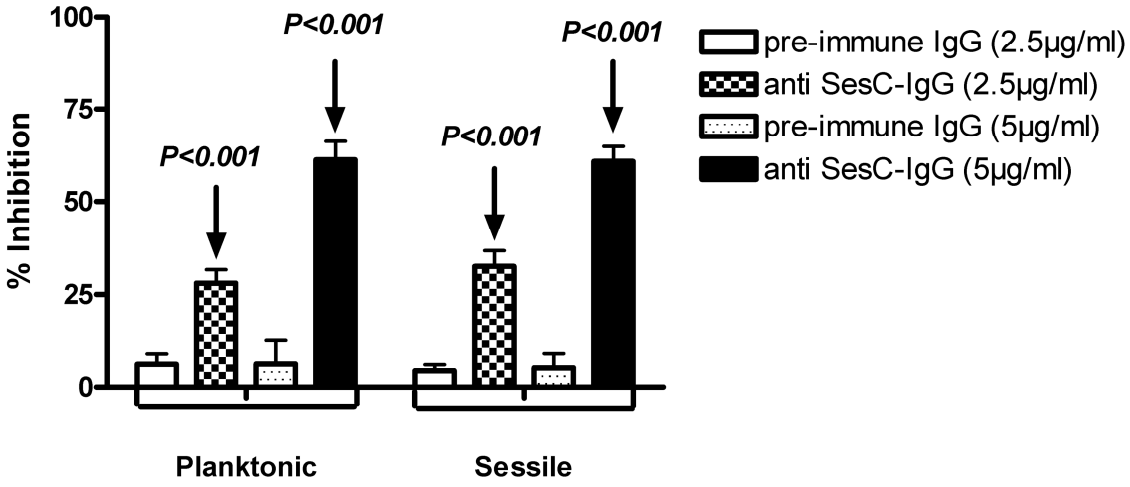


Figure 6.4. Effect of αSesC-IgGs on biofilm formation of *S. epidermidis* 10b bacteria from an overnight culture origin or isolated from an established (1-day) biofilm on catheter fragments. Formation of biofilm was measured with crystal violet stain. The error bars indicate standard deviations. Data are the average of 8 measurements in 2 independent experiments.

formation of a *sesC*-negative biofilm-positive *S. warneri* was non-significant, dose-independent and limited. αSesC-IgGs also showed activity against the most difficult-to-treat 1-day old biofilms *in vitro*. αSesC-IgGs could significantly reduce 1-day old biofilms of 10b on polystyrene microtiter plates *in vitro* in a dose-dependent manner.

Pintens *et al.* (personal communication-unpublished data) showed that *agr* mutations occur naturally in mature *S. epidermidis* biofilms at a relatively high rate, thus increasing the colonization and biofilm forming ability of these mutants. To investigate whether such spontaneous mutations occur in *sesC* in biofilms or whether biofilm formation by such *agr* mutants can be affected by αSesC-IgGs, the effect of αSesC-IgGs on biofilm formation of cells isolated from established biofilms was investigated. Data showed that αSesC-IgGs had the same effect on biofilm formation of cells isolated from an established biofilm as on biofilms formed by cells from an overnight culture.

Antibodies with complement proteins mediate opsonophagocytic killing of bacteria (Cerca *et al.* 2006). Another possible mechanism of action of antibodies is the inhibition of the function of a protein or ligand-receptor interactions which are caused by

conformational changes upon antigen binding (Brown and Koshland 1977;Einhauer and Jungbauer 2003;Oda *et al.* 2003). A possible mechanism for the effect of αSesC-IgGs on *S. epidermidis* biofilms in the absence of immune system components is probably that binding of αSesC-IgGs to SesC inhibits the function of SesC and thus biofilm formation.

These data are suggestive for; i) a possible role for SesC in *S. epidermidis* attachment to the polystyrene surface and biofilm formation, ii) potential use of SesC as a target antigen for inhibition of biofilm formation and treatment of established biofilms.

6.5. Conclusion

SesC might play a role in primary attachment of *S. epidermidis* to polystyrene surfaces and biofilm formation. SesC is a promising target for inhibition and treatment of *S. epidermidis* biofilms.

CHAPTER 7:

EFFECT OF SPECIFIC ANTI-SESC ANTIBODIES AND IMMUNIZATION WITH SESC ON *S. EPIDERMIDIS* BIOFILM FORMATION *IN VIVO*

7. Effect of specific anti-SesC antibodies and immunization with SesC on *S. epidermidis* biofilm formation *in vivo*

This chapter is based on the following reference: Mohammad Shahrooei, Vishal Hira, Rita Merckx, Benoit Stijlmans, Peter W.M. Hermans, Johan Van Eldere. 2009. Inhibition of *Staphylococcus epidermidis* biofilm formation by rabbit polyclonal antibodies against SesC protein. Infection and Immunity. 77(9):3670-8.

7.1. Introduction

So far, it has been shown that antibodies against the surface-exposed protein SesC can prevent the primary attachment, inhibit the accumulation phase and detach bacteria from an established *S. epidermidis* biofilm. These data demonstrating inhibition of biofilm formation with αSesC-IgGs *in vitro* indicate the relevance of SesC in *S. epidermidis* biofilm formation and the potential use of SesC as a promising target for vaccine development against *S. epidermidis* biofilms. However, expression and factors involved in biofilm formation *in vitro* and *in vivo* are different. For example in *in vivo* conditions, the surface of implants is rapidly coated by ECM proteins which are absent in *in vitro* conditions and are bound by different MSCRAMMs. These MSCRAMMs can promote adherence of bacteria to biotic surfaces.

In this chapter, using the rat model for FBI, the effect of anti-SesC antibodies (passive immunization) and immunization with SesC (active immunization) on *S. epidermidis* biofilm formation are investigated.

7.2. Materials and methods

7.2.1. Effect of αSesC-IgGs on 1-day old biofilms *in vivo*

For the *in vivo* inhibition assay, the rat model for *in vivo* catheter infection was used. Seven mm catheter fragments, pre-incubated for 20 min at 37°C with *S. epidermidis* 10b (≈10^4 cells/catheter) before implantation, were placed on ice and 8 fragments per rat were implanted immediately in EGF rats. After 24 h, the rats (9 rats divided into 3 groups

of 3 rats) were treated with 50 µg αSesC-IgGs diluted in PBS (total volume 330 µl), 50 µg pre-immune IgGs diluted in PBS (total volume 330 µl) or 330 µl of PBS via a subcutaneous injection at the place of catheter insertion.

Twenty-four hours after the injections, all 8 catheters from each rat were explanted and used for nucleic acid extraction as described (Vandecasteele *et al.* 2001;Vandecasteele *et al.* 2002). Real-time quantitative PCR of the guanylate monokinase housekeeping gene [*gmk* (SE0885: NP_764440)] was used to quantify the number of bacteria attached to the catheter fragments. Primers and probe for *gmk* were previously described (Pintens *et al.* 2008). As previously demonstrated (Vandecasteele *et al.* 2002), the number of *gmk* copies per catheter correlates very well with the number of CFU per catheter.

7.2.2. Effect of immunization of rats with rSesC on biofilm formation

For vaccination experiments, nine EGF rats were divided into 3 groups of 3 rats. Each rat in the first group was immunized twice (interval of 2 weeks) with respectively 100 and 50 µg rSesC (dissolved in normal saline) in CFA and IFA intraperitoneally. The second group of rats was injected with the same volume of normal saline in CFA and IFA but without antigen (rSesC). The third group was not immunized at all. The immune response after the second immunization of groups 1 and 2 were tested by ELISA of pre and post immunization sera samples of all rats. Subsequently, six catheter fragments pre-incubated with *S. epidermidis* 10b bacteria (≈10^4 cells/catheter) were implanted in each rat and 24 h later explanted and the numbers of sessile cells were quantified by CFU counting as previously described (Pintens *et al.* 2008). Briefly, after gentle cleaning with 0.9 % NaCl, catheters were placed in a tube containing 1 ml 0.9 % NaCl. Tubes were vortexed for 10 s, sonicated for 10 min at 40 kHz in a water bath (Branson 2200, Branson Ultrasonics) and again vortexed for 10 s. Thereafter, tube contents were diluted and 50 µl aliquots of 10-fold dilutions were plated on Tryptone Soya Agar (TSA, Oxoid) plates using a spiral plating system. Colonies on all plates were counted and the number of bacteria was defined as the mean of at least six quantitative cultures.

7.2.3. Statistical analysis

Statistical analyses were performed with GraphPad prism 4.2. A significant difference in adherence of bacteria to the catheter fragments between the controls and vaccine or treated groups with antibodies was tested with a one-way ANOVA.

7.3. Results

7.3.1. Effect of αSesC-IgGs on 1-day old biofilms *in vivo*

In the *in vivo* rat model, the number of sessile bacteria was quantified after one day by quantification of gDNA copies of the housekeeping gene *gmk*. Injection of 50 µg pre-immune IgGs reduced the number of biofilm-associated *S. epidermidis* 10b bacteria 0.64 $\log_{10}$ or 4-fold ($p<0,01$; 1-way ANOVA) whereas injection of 50 µg αSesC-IgGs decreased the number of biofilm-associated bacteria 1,78 $\log_{10}$ or 60.42-fold and 1.15 $\log_{10}$ or 14.35-fold ($p<0,001$; 1-way ANOVA) compared to the control group injected with PBS and the group injected with pre-immune IgG, respectively (Figure 7.1.).

7.3.2. Effect of immunization with rSesC on biofilm formation

The number of sessile bacteria attached to the catheter fragments 24 h after implantation in the immunized group with rSesC compared to normal saline-immunized and non-immunized control groups, was 1.1 $\log_{10}$ or 12.5-fold reduced ($p<0.01$; 1-way ANOVA). Data for each group were obtained from adherent bacteria to 18 catheter fragments implanted in 3 rats (Figure 7.2.).

7.4. Discussion

Data on the effect of passive and active immunization in our *in vivo* rat model for FBI are in line with our previous data on the effect of αSesC-IgGs on *S. epidermidis* biofilms *in vitro*. They indicate the relevance of SesC in *S. epidermidis* biofilm formation and the potential use of SesC as a target for vaccine development against *S. epidermidis* biofilms.

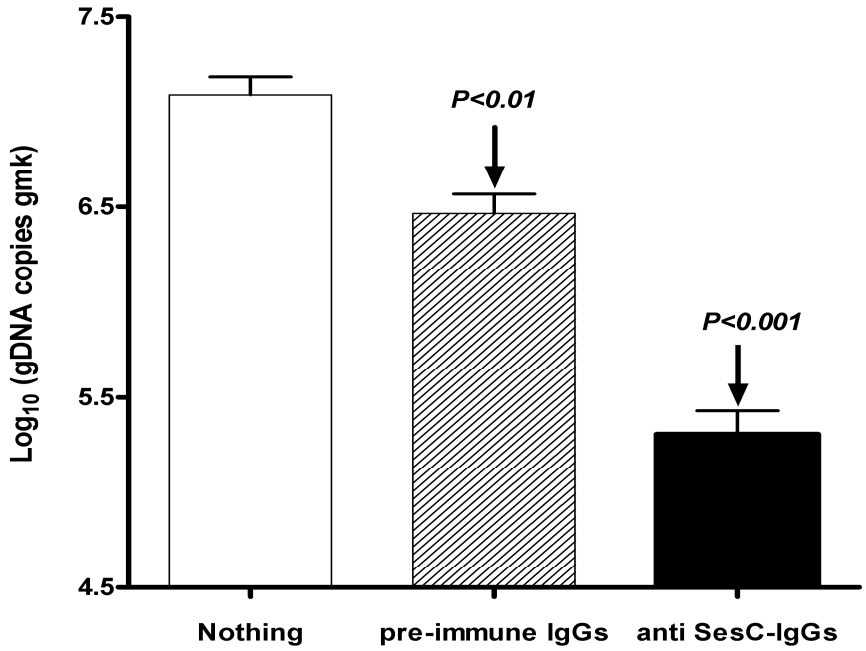


Figure 7.1. Effect of αSesC-IgGs on 1-day old biofilm of *S. epidermidis* 10b *in vivo*. One-day old biofilms on catheter fragments, implanted in 3 groups of rats (n=3 in each group), were subsequently treated with 50 µg pre-immune IgGs or αSesC-IgGs diluted in PBS (total volume 330 µl) or PBS via a subcutaneous injection at the place of catheter insertion. The next day, catheter fragments were explanted and the number of sessile bacteria was quantified by quantification of gDNA copies of the housekeeping gene *gmk*, represented on the Y axis. On the X axis the groups are indicated. The error bars represent standard deviations. Data for each group were obtained from adherent bacteria to 24 catheter fragments implanted in 3 rats.

The effect of αSesC-IgGs on *S. epidermidis* biofilms *in vitro*, in the absence of immune system components suggested a neutralizing effect for antibodies which block the function of SesC. Our preliminary data from an *in vitro* opsonophagocytosis assay suggest an opsonic activity of αSesC-IgGs (data not shown). However, these data were not statistically significant and the exact mechanism of action of antibodies on *S. epidermidis* biofilms *in vitro* and *in vivo* and the precise role of SesC in biofilm formation remains to be identified.

A technical limitation of our *in vivo* model is the quantification of the subcutaneous concentration of antibodies. The subcutaneous injection of 50 µg of αSesC-

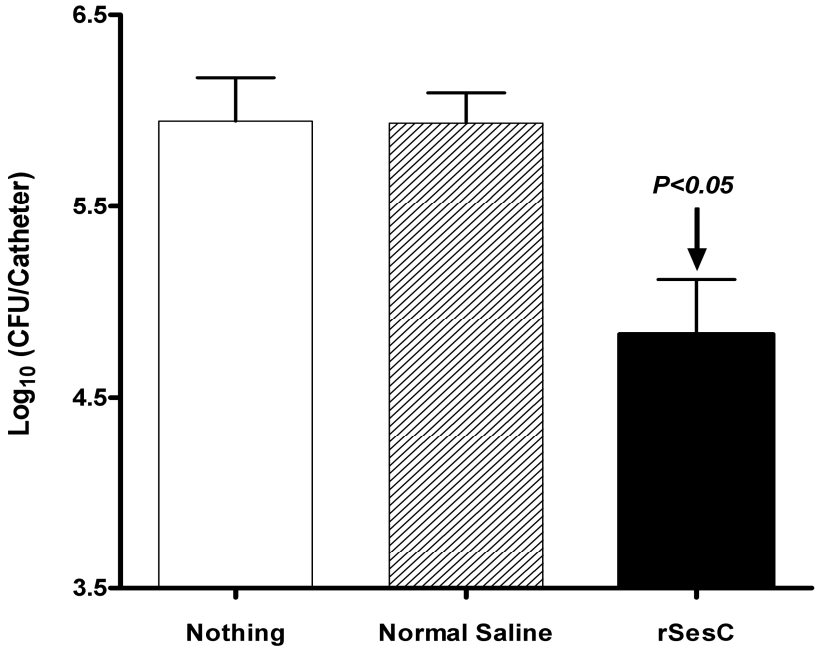


Figure 7.2. Effect of vaccination of EGF rats with rSesC on *S. epidermidis* 10b biofilm formation. The columns show the average load of bacteria on 18 catheter fragments 24 h after implantation. The error bars indicate standard deviations. Experiments were repeated twice.

IgGs at the place of insertion of the catheter fragments reduced the number of attached bacteria to the catheter fragments 1.78 $\log_{10}$ or 60.42-fold compared to the control group. In the vaccinated group compared to the controls the number of attached bacteria was reduced 1.1 $\log_{10}$ or 12.5-fold. A possible explanation for such difference (1.78 $\log_{10}$ or 60.42-fold vs. 1.1 $\log_{10}$ or 12.5-fold) between the effect of antibody injection and vaccination might be the higher concentration of antibodies at the place of infection in the passive immunization model.

7.5. Conclusion

In conclusion, immunoprophylaxis and immunotherapy targeting SesC might be a promising approach for prevention and treatment of *S. epidermidis* biofilms.

CHAPTER 8:

SESC STRUCTURE AND FUNCTION

8. SesC structure and function

This chapter is based on the following reference: Mohammad Shahrooei, Vishal Hira, Rita Merckx, Benoit Stijlmans, Peter W.M. Hermans, Johan Van Eldere. 2009. Inhibition of *Staphylococcus epidermidis* biofilm formation by rabbit polyclonal antibodies against SesC protein. Infection and Immunity. 77(9):3670-8.

8.1. Introduction

Although several genes in *S. epidermidis* have been mutated using allelic replacement (Pei and Flock 2001b;Tao *et al.* 2006;Vuong *et al.* 2000), transposon (Tn917) mutagenesis methods (Muller *et al.* 1993) or naturally occurring insertion of insertion sequences such as IS256 (Conlon *et al.* 2004), genetic manipulation of *S. epidermidis* is very difficult. For this reason we have a serious lack of knowledge about the basis of *S. epidermidis* virulence.

In silico prediction of the structure and function of a protein, could be a valuable approach if homologues of the target protein have been already characterized. However data obtained by *in silico* prediction should be subsequently confirmed by experimental data.

A complementary strategy which recently has been successfully used is transfer and expression of a gene in a surrogate bacterium. In this method a gene which encodes an unknown function protein is expressed in a strain that lacks that gene. Using this method, conversion of *S. epidermidis* strains from commensal to invasive was done by expression of the *ica* locus in strains lacking the *ica*-operon (Li *et al.* 2005) or *S. aureus* pathogenic genes were studied in the less virulent organism *Streptococcus gordonii* (Meier *et al.* 2001).

In this chapter, the experiments performed for unraveling the structure and function of SesC, are explained.

8.2. Materials and methods

8.2.1 *In silico* prediction of structure and function of SesC

Online bioinformatics tools were used to characterize SesC. To identify the possible repeat domains, the RADAR algorithm was used (http://www.ebi.ac.uk/Tools/Radar/index.html). A similarity and homology search was carried out using NCBI BLAST and FASTA (http://blast.ncbi.nlm.nih.gov/Blast.cgi, http://www.ebi.ac.uk/Tools/fasta33/). Protein secondary structure was predicted using PSIPRED server version 2.6 and CLC Combined Workbench version 3.6.2. (http://bioinf.cs.ucl.ac.uk/psipred/psiform.html, http://www.clcbio.com/index.php?id=93). Signal peptides were identified using the SignalP server (http://www.cbs.dtu.dk/services/SignalP-2.0/). Prediction of transmembrane helices was done using TMHMM server version 2.0 and SOSUI (http://www.cbs.dtu.dk/services/TMHMM-2.0/, http://bp.nuap.nagoya-u.ac.jp/sosui/sosui_submit.html). Secondary and tertiary structure was modeled using the 3D-PSSM Fold recognition server (http://www.sbg.bio.ic.ac.uk/~3dpssm/).

8.2.2. Construction of plasmids for *sesC::tet* allele replacement

In order to analyze the SesC function in *S. epidermidis,* we tried to replace *sesC* by a tetracycline resistance gene (*tet*) by homologous recombination. The upstream fragment (991 bp) was amplified using primer pairs US-F 5'-*GGTACC*AAATAGTCCTCCTTCGGTGATG-3' and US-R 5'-*GGGCCC*TTACACATACTCCTTTAATAAAGTTACCTG-3' with *Kpn*I and *Apa*I (*in italics and underlined*), respectively. The downstream fragment (902 bp) was amplified using primer pairs DS-F 5'-*GGATCC*TCTTGTTATAAACTTTGAGTAAGCGTT-3' and DS-R 5'-*CCCGGG*AATTGATGGTCTTGCATTAACTTC-3' with *Bam*HI and *Sma*I (*in italics and underlined*), respectively. The two fragments were cloned into the multiple cloning sites of temperature-sensitive *E. coli-Staphylococcus* shuttle vector pBT2 (Bruckner 1997), resulting in a plasmid termed pBTU-D. A fragment of 2606 bp containing the entire *tet*(M) gene was amplified by PCR from the pCN36 (Charpentier *et al.* 2004), using the primers tet-F 5'CTATGA*CCCGGG*AAATATTGAAGGCTAGTCAG-3' and tet-R 5'-GTTTAA*GGGCCC*CAAATATGCTCTTACGTGC-3' with *Sma*I and *ApaI* (*in italics and underlined*), respectively. The *tet*(M) gene was inserted into the vector pBTU-D, resulting in plasmid, pBT2Δ*sesC*. Following passage through the restriction-negative strain *S. aureus* RN4220, pBT2Δ*sesC* was

repurified and transformed into *S. epidermidis* RP62A by electroporation. Competent cells for electroporation were prepared by washing exponentially growing cells with 1% glycerol. Electroporation was performed at room temperature in a 0.2 cm cuvette (BTX Electroporation Cuvettes Plus^{TM}), at a setting of 2.5 kV, 25 µF, 100 ohm (Ω) for 2.5 ms using a gene pulser (BTX Electroporator ECM 630). The transformants obtained by electroporation were selected on BHI agar plates containing chloramphenicol (10 µg/ml) at 30°C. For gene replacement by homologous recombination, one transformant was cultivated in BHI with chloramphenicol (10 µg/ml) and tetracycline (5 µg/ml) at 30°C for 8 h and then with only tetracycline (5 µg/ml) at 43°C for 8 h. The culture was then diluted 50-fold in BHI and grown overnight without antibiotic. Appropriate dilutions of culture were plated on BHI agar plates containing tetracycline (5 µg/ml) and grown at 43°C. The colonies from tetracycline plates were replica plated both onto tetracycline and chloramphenicol + tetracycline plates. Colonies that could grow only on tetracycline plates, but not chloramphenicol + tetracycline, were selected as candidates for further testing. PCR amplification and sequencing were used for verification.

8.2.3. Cloning, expression and overexpression of *sesC* in *S. aureus* RN4220 and *S. epidermidis* RP62A

The entire coding region of the *sesC* gene of strain 10b was amplified using primers *sesC*-EF 5'-TACG*GGATCC*CAGGTAACTTTATTAAAGGAGTATGTGTAA-3' and *sesC*-ER 5'-ACGT*GGTACC*ACTAGAAGTTAATGCAAGACCATCAATTT-3' which incorporate flanking *Bam*HI and *Kpn*I restriction sites (*in italics and underlined*), respectively. The amplicon was ligated into pCN68 and pCN50 vectors (Charpentier *et al.* 2004), yielding pCN68*sesC* and pCN50*sesC*. The plasmids pCN68, pCN50, pCN68*sesC* and pCN50*sesC* were electroporated into *S. aureus* RN4220 yielding RN-pCN68, RN-pCN50, RN-pCN68*sesC* and RN-pCN50*sesC*, respectively. pCN50 and pCN50*sesC* were purified from RN4220 transformants, and electroporated into *S. epidermidis* RP62A yielding RP-pCN50 and RP-pCN50*sesC*, respectively. pCN68 compared to pCN50 has a higher copy number in *S. aureus*, contains the *ermC* gene (ribosomal methylase-encoding gene of pE194 for erythromycin resistance) instead of the *cat194* gene (chloramphenicol acetyltransferase-encoding gene of pC194 for

chloramphenicol resistance), and *sesC* in pCN68 is expressed under control of a constitutive promoter P*blaZ*. *sesC* gene expression in wild type and transformed strains was quantified by Taqman quantitative PCR. Briefly, overnight cultures of transformed strains and their parental strains in BHI were precipitated and washed once with PBS. The OD_{600} was adjusted to 1.0. Nucleic acid isolation and cDNA synthesis from 100 µl of each sample and quantification of both cDNA and genomic DNA were performed as previously described (Chapter 3.2.).

8.2.4. Biofilm formation by transformants

The amount of biofilm formed by RN- and RP-transformants and their parental strains was determined using a semi-quantitative adherence assay on 96-well polystyrene microtiter plates as previously described (Chapter 4.2.4.), in BHI medium or in BHI medium supplemented with 4% NaCl, 1% glucose or 3% ethanol to further induce biofilm development in RN4220 strain.

8.2.5. Adherence of transformants to immobilized Fg, Fn, Cn and von Willebrand factor in the presence and absence of αSesC-IgGs

Overnight cultures in BHI of transformed strains and their parental strains were precipitated and washed once with PBS. The OD_{600} was adjusted to 1.0 and the adherence was measured as follows. Wells of polystyrene microtiter plates were coated with human Fg (Sigma), Fn (Sigma), Cn (Sigma) and von Willebrand factor (VWF) (Sigma) in PBS overnight at concentrations ranging from 0.1 to 100 µg/ml. Blocking was done with 2% Bovine serum albumin (BSA) in PBS for 1 h at 37°C. After washing, either the pure cultures (100 µl per well) were pipetted into the plates or cultures were mixed with either pre-immune IgGs or αSesC-IgGs (5 µg/ml) and after 2 h of incubation at 4°C were added and allowed to adhere to the coated surfaces for 2 h at 37°C. After the incubation period, culture supernatants were washed and the remaining adherent cells were stained and quantified as previously described (Chapter 4.2.4.).

8.2.6. Statistical analysis

Statistical analyses were performed with GraphPad prism 4.2. as previously described (Chapter 6.2.5.).

8.3. Results

8.3.1. Subcellular localization, folding and structure prediction

SesC is predicted to encode a 676 aa protein with a predicted molecular mass of 75 kDa. The cytoplasmic precursor of SesC contains a 35 aa N-terminal signal peptide, a 37 aa C-terminal LPXTG sorting signal and a large extracellular domain. The N-terminal signal is required for sec-dependent secretion and cleaved by signal peptidase. The C-terminal signal is needed for cleavage between the threonine and the glycine of the LPXTG motif and attachment to peptidoglycan by sortase. One or two hydrophobic transmembrane helices and a positively charged cytoplasmic tail were identified within the N and C-terminus of the cytoplasmic precursor of SesC which are removed by signal peptidase and sortase. SesC is predicted to fold as either 30.9% α-helix and 1.2% β-sheets and 67.9% coil (PSIPRED prediction) or as 56.6% α-helix, 2.2% β-sheets and 41,2% coil (CLC prediction) (data not shown). Presence of mature SesC (≈68 kDa) in the cell wall fraction of *S. epidermidis* RP62A in the exponential and stationary phases of growth was shown using a Western immunoblotting technique (Bowden *et al.* 2005). In publicly available protein data banks, all homologues of SesC had less than 70% sequence identity to SesC and all homologues with identities higher than 26% were hypothetical proteins of unknown structure and function. The best homologue of SesC with a known function is a 341 aa fragment of the clumping factor A (ClfA) (26.6% identity, 65.1% similarity in 335-aa overlap). ClfA is a Fg-binding MSCRAMM of *S. aureus*. However, the putative Fg-binding site of ClfA is located outside this region of similarity.

8.3.2. Construction and isolation of a natural *S. epidermidis sesC* mutant

So far we have failed to knock-out *sesC* in *S. epidermidis* and we couldn't find a natural *S. epidermidis sesC*-mutant. More than 10000 colonies from tetracycline plates were replica plated both onto tetracycline and chloramphenicol + tetracycline plates,

however none of them was a tetracycline-resistant, chloramphenicol-sensitive colony. We collected 239 clinical and commensal CNS isolates from hospitalised patients (n=215) or from the skin of healthy individuals (n=24). 105 out of 239 isolates from patients and healthy persons were identified as *S. epidermidis* and 134 isolates as other CNS including *S. hominis* (n=17), *S. haemolyticus* (n=58), *S. warneri* (n=43), *S. capitis* (n=15), *S. saprophyticus* (n=1). All 105 *S. epidermidis* isolates were *sesC*-positive whereas non-*S. epidermidis* isolates were either *sesC*-negative (80%) or *sesC*-positive (20%).

8.3.3. Expression of *sesC* in *S. aureus* RN4220 and *S. epidermidis* RP62A

Quantitative PCR data show expression of *sesC* in RN-pCN50*sesC*, RN-pCN68*sesC* compared to their parental strain (RN4220) and overexpression of *sesC* in RP-pCN50*sesC* transformant compared to its parental strain (RP62A) (Figure 8.1.).

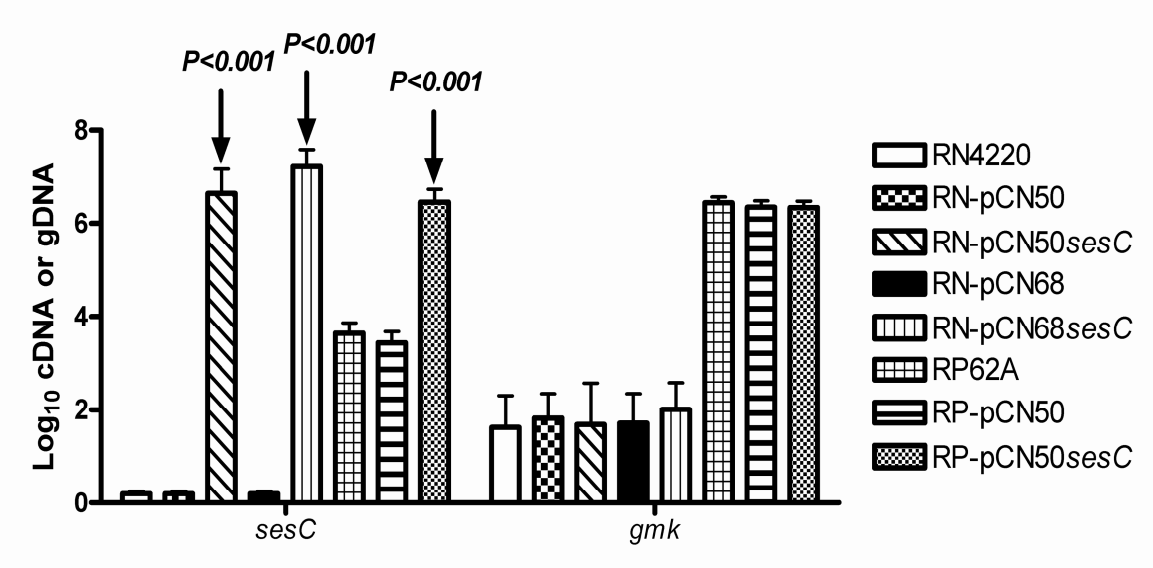


Figure 8.1. Expression of *sesC* in RN4220 and RP62A and their transformants. cDNA and gDNA were isolated from 100 µl of overnight cultures of each strain, and then expression of *sesC* and copy number of *gmk* as internal standard were measured by real time quantitative PCR. The error bars indicate standard deviations. Data are the average of 6 measurements in 2 independent experiments.

8.3.4. Effect of SesC expression on biofilm formation of transformants

Expression of SesC in RN4220 transformants completely inhibited biofilm formation in the presence of 4% NaCl but not 1% glucose or 3% ethanol, as biofilm inducers (Figure 8.2.). Overexpression of SesC had no effect on biofilm forming ability of RP62A (data not shown).

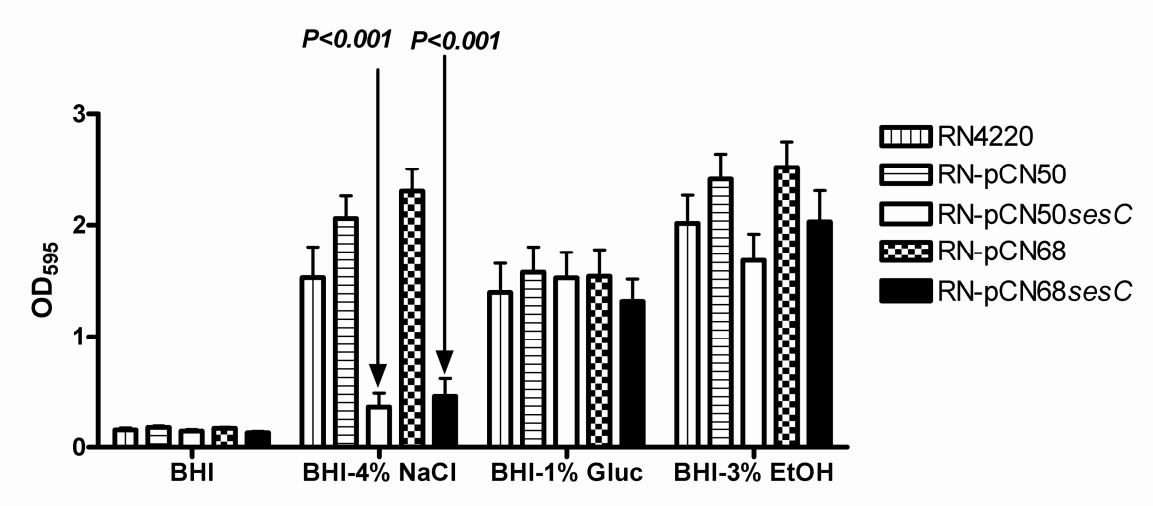


Figure 8.2. Comparison of the effect of sodium chloride (NaCl), glucose (Gluc) and ethanol (EtOH) on biofilm formation in RN4220 and its transformants. Overnight cultures of RN4220 and its transformants were diluted to an OD_{600} of 0.005 and grown in 96-well plates. Biofilm formation of the strains in BHI and in BHI+4% NaCl, BHI+1% Gluc, and BHI+3% EtOH was quantified at OD_{595} as explained. NaCl, glucose and ethanol can induce biofilm formation by RN4220. X and Y axes represent media (BHI medium or BHI medium supplemented with 4% NaCl, 1% Gluc, or 3% EtOH) and mean amount of biofilm (represented by OD_{595}), respectively. The error bars indicate standard deviations. Data are the average of at least 12 measurements in two independent experiments.

8.3.5. SesC is a potential Fg-binding MSCRAMM

To explore the function of SesC we expressed SesC in the *sesC*-negative *S. aureus* RN4220 and overexpressed sesC in the *sesC*-positive *S. epidermidis* RP62A by transformation of these strains with pCN68*sesC* and pCN50*sesC*. pCN68 has a higher copy number in *S. aureus* than pCN50. RN-transformants expressing SesC (RN-

pCN68*sesC* and RN-pCN50*sesC*) and the RP-transformant RP-pCN50*sesC* exhibited higher Fg-binding ability compared to their parental strains (Figure 8.3.). The binding level of these transformants to other host ECM proteins (Fn, Cn and VWF) was similar to the binding level of their parental strains (data not shown). RN-pCN68*sesC* had significantly higher fibrinogen-binding ability than RN4220 and RN-pCN68 ($p<0.001$ and $p<0.01$; 1-way ANOVA). RN-pCN50*sesC* and RP-pCN50*sesC* compared to their parental strains also exhibited significantly higher Fg-binding ability ($p<0.05$ and $p<0.01$; respectively; 1-way ANOVA). The Fg-binding ability of RN-pCN50*sesC* and RP-pCN50*sesC* was however not significantly different from their parental strains transformed by mock plasmids (RN-pCN50 and RP-pCN50). There was no significant difference between the Fg-binding levels of RN4220 or RP62A compared to RN-pCN50 or RP-pCN50, respectively.

Addition of αSesC-IgGs reduced the higher fibrinogen-binding ability of RN-pCN68*sesC* and RN-pCN50*sesC* to the wild type strain level and also reduced the Fg-binding ability of wild type and transformed RP strains to a lower level (Figure 8.3.). αSesC-IgGs had no effect on the binding ability of RN and RP wild types and transformed strains to Fn, Cn and VWF (data not shown).

8.4. Discussion

sesC encodes a protein which is translocated on the surface by its N-terminal signal and covalently anchored by sortase to peptidoglycan. Gene expression studies demonstrated expression of *sesC* in biofilm cells *in vitro* and *in vivo* which was confirmed by protein expression studies. The *in vitro* biofilm inhibition studies suggest a role for SesC in attachment to polystyrene surface in both primary attachment and accumulation phases. The *in vivo* biofilm inhibition studies showed that SesC plays also a role in attachment to the polyurethane surface (catheter), and also experimentally verified the potential use of SesC as a promising target for immunization against *S. epidermidis* biofilms.

The presence of this gene in all 105 tested *S. epidermidis* isolates confirms Bowden's report (Bowden *et al.* 2005) and is consistent with the failure of our efforts to

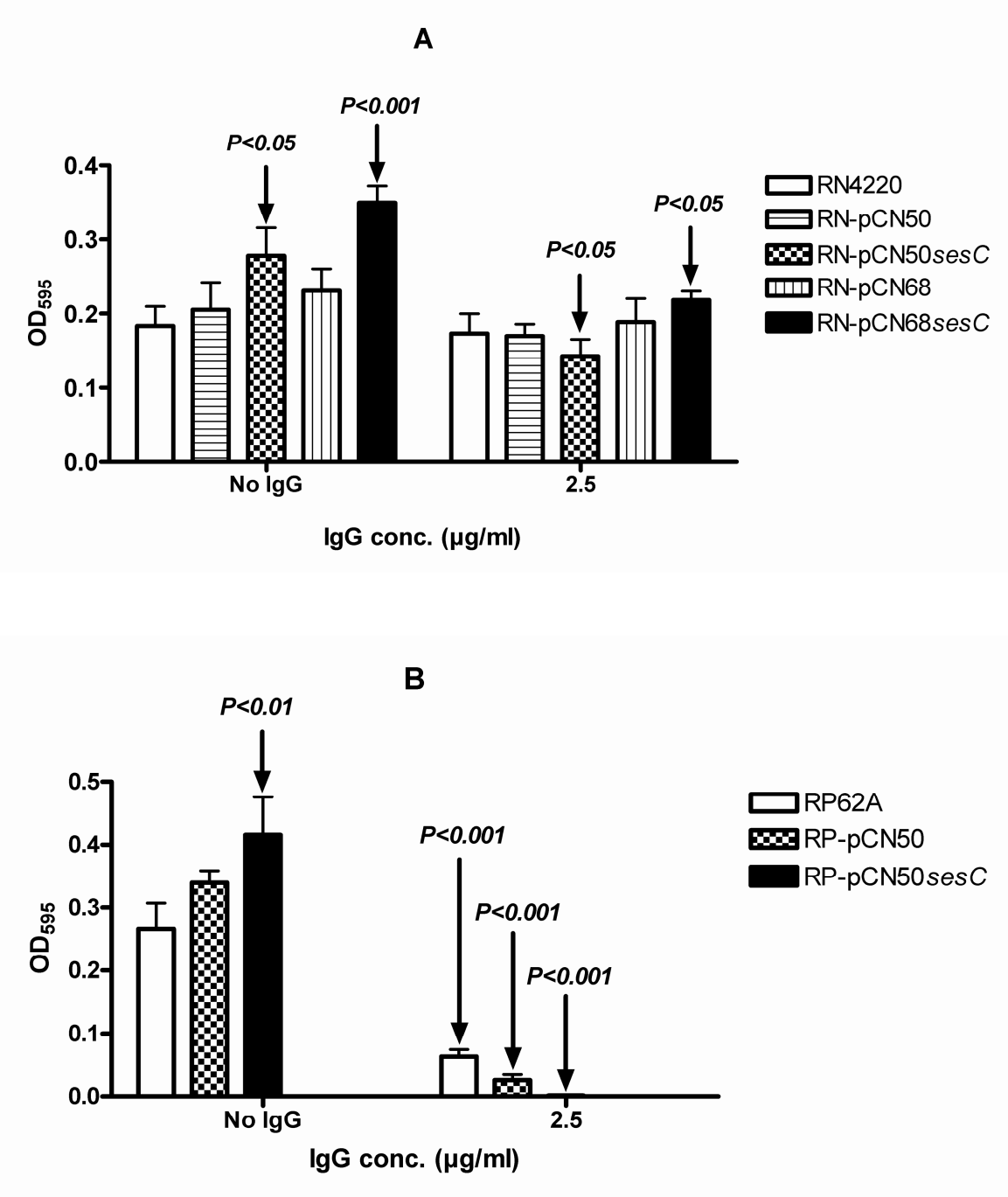


Figure 8.3. Fg-binding ability of RN4220 (A) and RP62A (B) transformants in the presence and absence of αSesC-IgGs. Ability of RN4220 and RP62A strains and their transformants to bind immobilized Fg was measured using plates coated with Fg. X and Y axes represent the concentration of αSesC-IgGs and OD_{595}, respectively. Binding activity for RN and RP strains was measured on plates coated with Fg at concentrations 10 and 1 µg/ml, respectively. The error bars indicate standard deviations. Data are average of 8 measurements in 2 independent experiments.

knock-out *sesC* in *S. epidermidis* indicating that *sesC* might be an essential gene. Yao *et al.*(Yao *et al.* 2005b) reported that *sesC* was absent in some *S. epidermidis* isolates, particularly in isolates from the skin of healthy individuals (9 out of 20 isolates). However, their data are doubtful since their data were generated based on transcriptome analysis and not identification of the gene by methods such as PCR amplification of the gene. Using other strategies such as antisense RNA technology or construction of a conditional mutant could be useful in future studies to identify the essential nature of *sesC*.

Since our efforts to knock-out *sesC* in *S. epidermidis* have failed and we couldn't find a natural *S. epidermidis sesC*-mutant, we expressed the gene in a *S. aureus sesC*-negative RN4220 which is an *ica*-positive and *ica*-dependent biofilm forming strain and also overexpressed *sesC* in *S. epidermidis* strain RP62A.

Our data show that *sesC* expression in RN4220 inhibits biofilm formation by this strain in the presence of NaCl as biofilm inducer but not when other biofilm formation inducers such as ethanol and glucose are used. There are several explanations for this phenomenon. The first explanation is that the expression of SesC switches the *ica*-dependent mechanism of biofilm formation in RN4220 to the *ica*-independent mechanism of biofilm formation in RN-transformants. Several studies have shown that *ica*-positive *S. aureus* and *S. epidermidis* strains can switch from the *ica*-dependent to the *ica*-independent mechanism of biofilm formation which can be inhibited by NaCl (Fitzpatrick *et al.* 2005;Hennig *et al.* 2007). We found a natural *S. aureus* among the clinical isolates which is an *ica*-positive, *sesC*-positive strain, and in which biofilm formation can be inhibited by NaCl similar to what we observed in the RN-transformants expressing SesC (data not shown). However, at the moment there are no other experimental data to support this assumption.

In RP-transformants, the expression of *sesC* on the chromosome of bacteria might be downregulated in response to the constitutive expression of the *sesC* gene on plasmid. This might explain why biofilm formation in RP-transformants and the parental strain is not different. Constitutive, high expression of *sesC*, which encodes the LPXTG protein SesC might lead to the overexpression of SesC and consequently competition with other LPXTG proteins for location on the surface. The presence of these other LPXTG proteins

might be necessary for biofilm development in the presence of NaCl. However, biofilm formation of RN-transformants in the presence of other inducers (glucose and ethanol) was similar to their parental strain.

Expression of *sesC* in the *sesC*-negative *S. aureus* RN4220 or overexpression of *sesC* in the s*esC*-positive *S. epidermidis* RP62A did lead to a clear effect on Fg-binding, but not to other host ECM proteins. However, the rSesC didn't show any fibrinogen-binding activity (data not shown). αSesC-IgGs could restore the Fg-binding level of the RN-transformants to the Fg-binding level of their parental strain and significantly reduced the Fg-binding ability of RP62A and its transformants but showed no effect on Cn, Fn and VWF-binding ability of tested strains. These data suggest that SesC can act as a Fg-binding MSCRAMM and αSesC-IgGs efficiently and specifically inhibit the SesC function and have no effect on the function of other MSCRAMMs.

Gene expression data support the involvement of SesC in the accumulation phase and persistence of biofilm, whereas biofilm inhibition studies show its involvement in primary attachment to naked and Fg-coated surfaces of polystyrene microtiter plates as well. An example for such a protein which plays a role in attachment to abiotic surfaces and also shows host matrix protein-binding activity is AtlE (Heilmann *et al.* 1997).

8.5. Conclusion

In conclusion, SesC might be a potential Fg-binding MSCRAMM, which plays a role in attachment to abiotic surfaces. Data from this part of the study are in line with other data and support a role for SesC in *S. epidermidis* biofim formation. However, further investigation is necessary to characterize the structure and analyse the function of SesC.

CHAPTER 9:

GENERAL CONCLUSION AND FUTURE PERSPECTIVES

9. General conclusion and future perspectives

9.1. General conclusion

The following considerations highlight the need for vaccine development and prophylactic measures against *S. epidermidis* infections.

Compared with *S. aureus*, *S. epidermidis* does not produce as many toxins and tissue-damaging exoenzymes and *S. epidermidis* infections may rarely become life threatening. However, *S. epidermidis* is now considered as the major cause of device-related infections, infections which have increased in number, owing to the increased use of such devices. Besides, due to the formation of biofilm such infections are notoriously difficult to treat and currently, the only effective method for curing biofilm infections is to remove and replace the infected device, placing the patient at increased risk for complications due to these additional procedures.

There is evidence suggesting that *S. epidermidis* can transfer genes to *S. aureus* (Hanssen *et al.* 2004). Since *S. epidermidis* is a ubiquitous human skin commensal with high frequency of antibiotic resistance, it can act as a reservoir and carrier of antibiotic resistance genes to *S. aureus*. On the other hand, although so far this gene transfer appears to be a one-way street, it suggests that there is the possibility that virulence genes may be transferred from *S. aureus* to *S. epidermidis*. Gene transfer between *S. aureus* and *S. epidermidis* may have an enormous impact on public health.

Currently, there is no anti-*S. epidermidis* vaccine. Nabi's EpiVAX™ is a conjugate vaccine (patented antigens, PS-1 and GP-1) which entered into phase I clinical trials. Other research groups (Guss and Flock, U.S. Patent Application 20090214584) also patented other antigens as potential vaccine targets against *S. epidermidis* infections. However, no more data are available.

In this study, using reverse vaccinology, SesC was identified as the best potential antigen for vaccination and production of protective antibodies against *S. epidermidis* in FBI. Characteristics of SesC as the best target are:

1. *sesC* gene was present in all tested *S. epidermidis* isolates, indicating its possible essential role in *S. epidermidis.*

2. SesC protein is a surface-exposed protein which is expressed in both planktonic and sessile bacteria *in vitro* and *in vivo.*

3. *sesC* is highly expressed during the early and late phases of FBI, indicating its possible importance to pathogenicity.

4. Rabbit polyclonal αSesC-IgG antibodies could prevent adherence of *S. epidermidis* to polystyrene surface, indicating its possible role in colonization of abiotic surface.

5. αSesC-IgGs could inhibit biofilm formation of *S. epidermidis in vitro* in polystyrene plates during an overnight incubation, indicating its possible role in accumulation and maturation of biofilms.

6. αSesC-IgGs could destabilize established (1-day old) biofilms, indicating its possible role in establishment of *S. epidermidis* biofilms.

7. Subcutaneous injection of αSesC-IgGs at the insertion place of the catheters fragments could significantly reduce the number of attached bacteria to the catheter fragments in 1-day old biofilms, indicating the possible application of αSesC-IgGs for the treatment of established biofilms.

8. Vaccination with SesC could elicit protective immunity in rat animal model for FBI, indicating its potential as a vaccine antigen.

9. αSesC-IgGs had no effect on biofilm formation of a *ses*-negative clinical isolate of *S. warneri*, indicating the specificity of the binding of antibodies to SesC.

10. *S. aureus* RN4220 transformants expressing SesC or RP62A transformants overexpressing SesC could bind Fg and not other ECM proteins better than their parental strains. The higher Fg-binding level of transformants could be restored to the parental Fg-binding level by αSesC-IgGs, indicating the specificity of the binding of antibodies to SesC and the fact that SesC might be a potential Fg-binding MSCRAMM.

11. In the presence of sodium chloride but not other biofilm inducers such as ethanol and glucose, *S. aureus* RN4220 transformants expressing SesC couldn't form biofilm compared to the parental strain, suggesting that mechanisms of biofilm formation in transformants and their parental strain might be different or that expression of SesC inhibits the effect of NaCl on *ica* operon expression.

Several studies have shown that there are two mechanisms (*ica* or PIA-dependent and *ica*-independent or proteinaceous) of biofilm formation in *S. epidermidis*. Although the *ica* or PIA-dependent mechanism of biofilm formation is currently the best-understood mechanism, recent studies have shown the presence of *S. epidermidis* and *S. aureus* strains which possess and express the *ica* operon, but are incapable of biofilm development or that switch from the *ica*-dependent to *ica*-independent biofilm development mechanism (Fitzpatrick *et al.* 2005;Hennig *et al.* 2007).

By examining the environmental regulation of biofilm development it has been demonstrated that sodium chloride is an activator of *ica* operon transcription and involves the production of PIA/PNAG, whereas it can't induce the proteinaceous mechanism of biofilm formation (Conlon *et al.* 2004;Fitzpatrick *et al.* 2005;Hennig *et al.* 2007;O'Neill *et al.* 2007). However, the biofilm formation of strains which switch from *ica*-dependent to *ica*-independent or those which develop a biofilm by the *ica*-independent mechanism can be induced by environmental regulators such as glucose and ethanol (Fitzpatrick *et al.* 2005;Hennig *et al.* 2007).

We have recently studied biofilm formation of 58 clinical isolates of *Staphylococcus* spp. in BHI or BHI supplemented with 4% NaCl, 1% glucose, or 3% ethanol (data not shown). We found a *sesC*-positive, *ica*-positive *S. aureus* isolate in which the biofilm formation can be induced by glucose but inhibited by sodium chloride similarly to biofilm formation in the RN-transformant expressing SesC (data not shown). These data support our hypothesis, that there might be a link between SesC and *ica*-independent biofilm formation in *S. aureus*.

Our data show the involvement of SesC in attachment to naked and Fg-coated surfaces of polystyrene microtiter plates. An example for such a protein which plays a role in attachment to abiotic surfaces and also shows host matrix protein-binding activity is AtlE (Heilmann *et al.* 1996;Heilmann *et al.* 1997).

All these data are in line and support each other, suggesting that SesC is a promising antigen for vaccination against *S. epidermidis* biofilm formation and production of protective antibodies against *S. epidermidis* established biofilms. However, the precise role of SesC in biofilm formation and the mechanism of function of αSesC-IgG antibodies *in vivo* remain to be identified.

Preliminary results from an *in vitro* opsonophagocytosis assays which mimic the *in vivo* defense strategy suggest an opsonic activity of αSesC-IgGs (data not shown). Prevention of initial attachment and biofilm formation *in vitro* by αSesC-IgG in the absence of immune system components suggests a neutralizing effect which means probably binding of αSesC-IgGs to SesC inhibits the function of SesC and thus biofilm formation. Incubation of bacteria with αSesC-IgG had no effect on bacterial growth.

9.2. Future perspectives

Figure 9.1. shows different steps of reverse vaccinology (Mora *et al.* 2003), starting from *in silico* prediction of potential antigens and ending with vaccine development.

Future investigations are necessary:

1. To unravel the precise structure and function of SesC. Construction of a *S.*

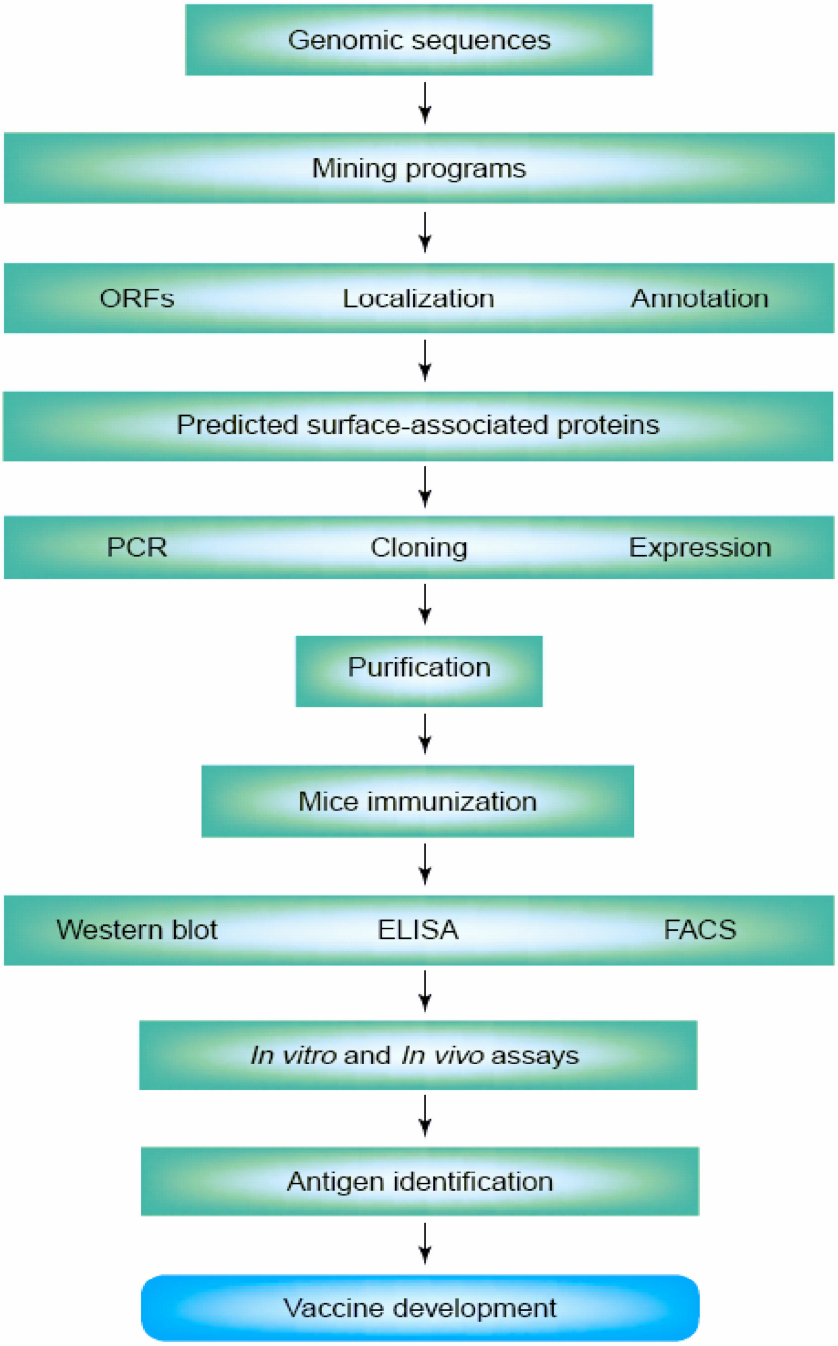


Figure 9.1. Flow chart of the genome-based approach to vaccine development. This approach involves the *in silico* analysis of microbial genome sequences followed by the high-throughput expression of the genes of interest. The recombinant proteins are then used to immunize animals and the post-immunization sera are analyzed to assess the ability of the polypeptide to elicit a quantitative and qualitative immune response (Mora *et al.* 2003).

epidermidis sesC-conditional mutants or methods such as antisense RNA technology can help us to unravel the role of SesC in biofilm formation. Knowing the protein's structure and function opens the door to design more efficient strategies to target its function.

2. The antigenic epitopes of SesC must be identified and tested individually. Knowing the function and structure of SesC can help us to design and use short peptides that contain the active site of protein for immunization.

3. Since SesC was selected as the best antigen among five candidates only based on an *in vitro* assay, it might be worth to test the potential use of the other 4 candidates for biofilm inhibition *in vivo*. If immunization with other proteins shows any anti-biofilm activity, a short peptide can be designed containing different epitopes from different proteins which most likely elicits higher protection compared to a single protein as a target.

Our *in vivo* rat model, although closely resembling the subcutaneous models for FBI and mimicking intra-operative contamination with skin flora, doesn't mimic conditions found in the human intravascular system. Intravascular devices are nevertheless the most frequently used medical devices. In addition, the immune response at the site of infection in our subcutaneous rat model may not reflect the immune response to the intravascular device-related infections in peripheral blood. Hence, it might be worthwhile to investigate the immune response in other rat FBI models for example a model that mimics intravascular conditions (Rupp and Fey 2001;Ulphani and Rupp 1999).

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CURRICULUM VITAE

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Lab Assistant Student, Shiraz University, Biology Department, Shiraz, Iran (1997 - 1999)

Research Assistant and Medical Lab Assistant, National Research Center for Genetic Engineering and Biotechnology (NRCGEB), Tehran, Iran (1999 - 2002)

Research Fellow, Department of Molecular Genetics VIB8, University of Antwerp, B - 2610 Antwerp, Belgium (January 2003 - June 2003)

CONFERENCE PRESENTATIONS

LPXTG protein SesC: a newly identified cell wall-anchored protein involved in *Staphylococcus epidermidis* biofilm formation

Mohammad Shahrooei, Vishal Hira, Peter W.M. Hermans, Johan Van Eldere

47th Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC), September 17-20, 2007, Chicago, U.S.A (**Poster** Presentation)

The possible role of the LPXTG protein SesC in *Staphylococcus epidermidis* biofilm formation

Mohammad Shahrooei, Vishal Hira, Rita Merckx, Benoit Stijlmans, Peter W.M. Hermans, Johan Van Eldere

18th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID), April 19-22, 2008, Barcelona, Spain (**Poster** Presentation)

Role of SesC in *Staphylococcus epidermidis* biofilm formation

Mohammad Shahrooei, Vishal Hira, Rita Merckx, Benoit Stijlmans, Peter W.M. Hermans, Johan Van Eldere

48th ICAAC/IDSA 46th Annual Meeting (48th Annual Interscience Conference on Antimicrobial Agents and Chemotherapy/ 46th Annual Meeting of the Infectious Diseases Society of America**,** October 25-28, 2008. Washington, DC, U.S.A (**Poster** Presentation)

Immunization against SesC reduces *Staphylococcus epidermidis* biofilm formation

Mohammad Shahrooei, Vishal Hira, Rita Merckx, Benoit Stijlmans, Sona Kucharikova, Patrick Van Dijck Peter W.M. Hermans, Johan Van Eldere

19th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID), April 16-19, 2009, Helsinki, Finland (**Oral** Presentation)

Sterilization of staphylococcal biofilms with delta-toxin plus Rifampin in a rat model

Valerie Pintens, **Mohammad Shahrooei,** Rita Merckx , Johan Van Eldere

19th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID), April 16-19, 2009, Helsinki, Finland (**Poster** Presentation)

Potential vaccine targets in *Staphylococcus epidermidis*

Mohammad Shahrooei, Vishal Hira, Rita Merckx, Benoit Stijlmans, Peter W.M. Hermans, Johan Van Eldere

49th Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC), September 12-15, 2009, San Francisco, U.S.A (**Oral** Presentation)

Rapid quantitative monitoring of mixed chimerism using amplification a highly discriminative PCR-STR system after bone marrow transplant

Mohammad Shahrooei, Ahmad Aleyasin, Katayon Darvishi, Kamran Alimoghadam, Ardeshir Ghavamzadeh

European Human Genetics Conference 2002 (ESHG), May 25-28, 2002, Strasbourg, France (**Poster** Presentation)

Genetic fingerprinting of patients after allogeneic bone marrow transplantation using recipient mouthwash samples

Mohammad Shahrooei, Ahmad Aleyasin, Kamran Alimoghadam, Ardeshir Ghavamzadeh

52nd Annual Meeting of The American Society of Human Genetics 2002, October 14-18, 2002, Baltimore, Maryland, USA (**Poster** Presentation)

PUBLICATIONS

Inhibition of *Staphylococcus epidermidis* biofilm formation by rabbit polyclonal antibodies against SesC protein

Mohammad Shahrooei, Vishal Hira, Rita Merckx, Benoit Stijlmans, Peter W.M. Hermans, Johan Van Eldere

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